CAROTENOID ACCUMULATION DURING GRAIN DEVELOPMENT IN DURUM WHEAT (*TRITICUM TURGIDUM* L. VAR. *DURUM*)

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ABSTRACT

Yellow pigment (YP) concentration is an important quality trait in durum wheat (*Triticum turgidum* L. var *durum*) and is comprised primarily of carotenoids. The main objective of our study was to measure the accumulation of carotenoids during the grain fill period to improve our understanding of the physiological basis for differences among durum wheat cultivars. Thirteen cultivars and breeding genotypes with large variation in total YP concentration (<6 μg g^{-1} to >15 μg g^{-1}) were studied. Spikes were sampled from replicated field plots in 2007 and 2008 near Saskatoon and Swift Current, Saskatchewan, Canada, at 14, 21, 28 and 35 days after heading (DAH). The remainder of each plot was combined at grain maturity for YP and carotenoid analysis. Carotenoids were extracted with 1:1 methanol:dichloromethane (0.1% BHT) and quantified with HPLC. *Trans* (*E*)-lutein was the predominant carotenoid at maturity and was detected at 14 DAH in all genotypes. The rate and duration of *E*-lutein accumulation was variable among genotypes expressing high, intermediate and low YP. The accumulation of all carotenoids was lowest in genotypes expressing low YP, and suggests rate limitations early in the carotenoid biosynthetic pathway. *E*-zeaxanthin concentrations were highest in mature grain, but no significant differences were detected among genotypes. However, the ratio of *E*-zeaxanthin to *E*-lutein was inversely correlated with total YP, suggesting that the β,ε branch of lycopene cyclization is favoured over the β,β branch in high-YP genotypes. These results provide insights to the regulation of the carotenoid biosynthetic pathway during grain fill stage in durum wheat and will facilitate breeding for higher carotenoid concentration.

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1.0 INTRODUCTION

Elevated yellow pigment (YP) concentration is a desirable end-use quality trait in durum wheat (*Triticum turgidum* L. var *durum*) and is an important target of durum breeding programs worldwide (Cenci et al., 2004, Elouafi et al., 2001). Endosperm yellow colour is valued in the semolina and pasta industry for its consumer appeal and for the health benefits associated with carotenoids, such as antioxidant activity and prevention of macular degeneration (Abdel-Aal et al., 2007). In processed durum products, the degree of yellowness is influenced by several factors, including the presence of carotenoid pigments (Hentschel et al. 2002; Panfili et al. 2004), semolina extraction rate (Matsuo and Dexter 1980), processing conditions (Borrelli et al. 1999), and oxidative degradation by lipoxygenases (LOX) (Borrelli et al. 1999).

The major carotenoid in durum wheat endosperm is *trans*(*E*)-lutein (Abdel-Aal et al., 2007, Panfili et al., 2004). In a study involving durum, bread wheat (*T. aestivum* L.), emmer and einkorn (*T. monococcum* L.), a high correlationwas found between lutein and YP (r=0.94) (Abdel-Aal et al., 2007). Similarly, a high correlation (r = 0.98) was reported in durum wheat between lutein and YP (Fratianni et al., 2005) . Carotenoids are found throughout the kernel in durum and related species, including einkorn wheat and bread wheat (Adom et al., 2005; Hentschel et al., 2002; Hidalgo and Brandolini, 2008). In addition to lutein, traces of zeaxanthin and β-carotene have been reported in durum endosperm (Abdel-Aal et al., 2007, Leenhardt et al., 2006, Panfili et al., 2004), and β-cryptoxanthin has been reported in einkorn (Hidalgo and Brandolini, 2008).

The genetics of YP concentration in durum wheat has been extensively studied and is largely controlled by genetic factors with additive effects (Elouafi et al., 2001; Clarke et al. 2006). Major quantitative trait loci (QTL) for YP have been found on the group 2 chromosomes (Joppa and Williams, 1988; Pozniak et al., 2007) chromosomes 6B and 7B (Pozniak et al., 2007), and 3B (Patil et al., 2008). Recently, two linked QTL for YP were reported on 7A (Singh et al. 2009). In hexaploid wheat, QTLs have been found on chromosomes 3A and 7A (Parker et al., 1998). The gene *Psy1* (coding for phytoene synthase) has been indicated as a candidate gene for the 7A (Elouafi et al., 2001; Singh et al., 2009) and 7B QTLs (Pozniak et al., 2007).

The genetics behind carotenoid accumulation have been characterized in depth in other plants; however, little is known about the regulation of this pathway in durum wheat. Therefore, this study was designed to profile carotenoid accumulation during the grain fill period among genetically variable durum cultivars and breeding lines. This will help in understanding how the pathway is regulated, and why YP concentrations vary among genotypes.

2.0 LITERATURE REVIEW

2.1 Durum Wheat

Durum wheat (*Triticum turgidum* L. var *durum*), 2n=28, genome AABB, is in the family Poaceae, tribe Triticeae. It evolved in the Middle East via interspecific hybridization and chromosome doubling followed by domestication. The parent species, *T. monococcum* and *Aegilops speltoides*, contributed the A and B genomes respectively (Bozzini, 1988). It is more drought tolerant than hexaploid bread wheat (*T. aestivum*) and has the hardest kernel of all wheats (Elias and Manthey, 2005). Worldwide durum production in 2007 was about 33 MT, of which about 10% was in Canada. Over 50% of the worldwide durum export market share is held by Canada; the main importers are North Africa and the European Union, both of which are net importers of durum wheat (Morgan, 2007). Up to 70% of Canadian durum is exported, indicating the importance of focusing on buyer needs (Dexter and Marchylo, 2001). Because durum is considered a type of specialty wheat and is generally not interchangeable with bread wheat in food products, its demand is inelastic (AAFC, 2007). Ideally, durum wheat has a hard, vitreous kernel, a yellow endosperm (AAFC, 2007) and a grain protein content ranging from about 12.8% to 15.7% (Del Moral et al., 2007). In Canada, Canadian Western Amber Durum (CWAD) is the market class and is dominated by the cultivar Strongfield (Canadian Wheat Board, 2010). Durum wheat is primarily milled to produce semolina, which is used for producing couscous and pasta. Pasta is made from durum semolina (150-450 μm particle size) and water that is formed into dough and extruded under vacuum through a die. Although durum is also made into bread in some parts of the world, this is a relatively small proportion of worldwide durum usage (Elias and Manthey, 2005). Current breeding objectives in the Canadian durum industry are higher yellow pigment and gluten strength, reduced grain cadmium concentration and increased fusarium head blight resistance (Clarke et al., 2009; Dexter and Marchylo, 2001).

2.2 Yellow pigment in durum wheat grain

2.2.1 Importance of yellow pigment

Yellow pigment (YP) in durum wheat endosperm, primarily caused by carotenoids, is an important processing quality trait that contributes to semolina yellow colour (Cenci et al., 2004, Elouafi et al., 2001) and is essential for most pasta products as dictated by consumer demand (Atienza et al., 2007). In addition, the carotenoid components of YP possess numerous health benefits (section 2.3.6). Pasta colour, which has yellow, brown and occasionally red components (reviewed by Ruiz et al., 2005) is increasing in importance as durum breeding programs are focusing on higher semolina extraction rates (Dexter and Marchylo, 2001). Although most consumers continue to favour increased pasta yellowness, there may be a maximum limit in some markets because of consumer aversion to perceived artificial colourants (Dexter and Marchylo, 2001).

2.2.2 Genetic and environmental control

YP is a highly heritable trait controlled by additive gene effects, which makes it appropriate to select for in early generations of breeding programs (Joppa and Williams, 1988). The heritability of YP concentration is high and ranges from 0.79 to 0.94 (reviewed by Clarke et al., 2006). Elouafi et al. (2001) measured heritabilities ranging from 0.48 to 0.99 in 16 crosses while Clarke et al. (2006) reported values ranging from 0.34 to 0.95. Lower heritability values were reported in unreplicated, single location trials which generally have low statistical power. Nachit et al. (1995) found heritabilities of YP to range between 0.90 and 0.97. Transgressive segregation of semolina yellow colour has been reported in various studies (Elouafi et al., 2001; Johnston et al., 1983), suggesting many genetic factors contribute to YP expression. Elouafi et al. (2001) found significant QTLs associated with YP on chromosomes 7AL and 7BL in a RIL mapping population. Major genes have also been reported on the group 2 chromosomes (Joppa and Williams, 1988). Minor QTL have been reported on chromosomes 4A and 5A (Hessler et al., 2002), 3A and 7A in hexaploid wheat (Parker et al., 1998) and on 3BS (Mares and Campbell, 2001; Patil et al., 2008). Significant QTLs were again reported on chromosomes 2A, 4B, 6B and 7B in a doubled haploid mapping population (Pozniak et

al., 2007), with the 7B QTL having the largest effect. At least three genetic factors were found to influence semolina YP in all environmental cross combinations, although the median number of factors was 6-7 (Clarke et al., 2006).

Pasta colour is also dependent on other factors, such as the LOX activity during processing (Hessler et al., 2002), semolina extraction rates, and pasta drying conditions (Dexter and Marchylo, 2001). LOX are non-haeme, iron containing deoxygenase enzymes that mediate the hydroperoxidation of 1,4-cis, cis pentadiene structures in free fatty acids, particularly linoleic acid (18:2) (Trono et al., 1999). These hydroperoxides may then oxidize the carotenoid components of YP. The prior presence of free fatty acids is required, as LOX do not react with triacylglycerols, nor is there significant lipase activity during pasta manufacture (Trono et al, 1999). LOX is inhibited by β-carotene (Troccoli et al., 2000), a minor carotenoid in semolina (Abdel-Aal et al., 2007). LOX activity has a moderate heritability as it is transcriptionally and environmentally regulated (Troccoli et al., 2000). As these enzymes possess significant functionality up to 60°C (Barone et al., 1999), the use of ultra-high temperature (UHT) drying maintains pasta yellow colour and also limits the formation of undesirable brown pigments formed by the action of peroxidases on carotenoids (Troccoli et al., 2000).

YP is, by convention, reported as a concentration on a 14% moisture basis in durum flour, meal or semolina (AACC 14-50). The effects of genetic and environmental conditions on kernel weight as well as on carotenoid synthesis and/or breakdown (Clarke et al., 2006) can directly or indirectly influence YP concentration in the kernel. Hessler et al. (2002) found that the increased starch content of large seeds, which can be approximated by kernel weight, had a diluting effect on the semolina YP concentration. Guler (2003) reported a significant positive effect of irrigation on increasing kernel weight, on average, with highest kernel weights occurring with moderate (200 mm) and high (300 mm) irrigation. However, the average YP concentration of semolina was highest with moderate irrigation, indicating that the increased starch content may have been fully compensated for by an increased content of carotenoids or other pigments in the semolina. Mangels (1932) reviewed by Clarke et al. (2006), also found increased YP content in grain grown under cool, wet conditions. Similarly, Lee et al. (1976) found

that there was greater heterosis in the concentration of YP when hybrids were grown under relatively cool, wet conditions. On the contrary, other studies have found results that are more in agreement with the inferences drawn by Hessler et al. (2002). Clarke et al. (2006) found variable and weak negative correlations between kernel weight and semolina YP content, indicating other, more important factors affecting YP. The authors also found a weak correlation between warm, wet growing season conditions and semolina YP content. Alvarez et al. (1999) found a significant negative correlation of -0.59 between kernel weight and YP content. All these results indicate that a complex set of genetic and environmental factors influence the final YP content of semolina, despite the relatively high heritabilities that are usually estimated.

2.2.3 Analysis and Composition

Total YP is typically assayed spectrophotometrically with AACC method 14-50 or ICC method 152 (Hentschel et al., 2002). By convention, absorbances are read at 437 nm and calculated using the extinction coefficient for β-carotene. Both are characterized by the use of water-saturated *n*-butanol as the extraction solvent, the low volatility of which enables reasonably accurate analysis after extraction and filtration. The inclusion of water is to generate clear, clarified extracts that can be filtered without centrifugation (Binnington et al., 1938). However, recent research indicates that the presence of about 20% water in this solvent may also help in improving the extraction recovery of carotenoids (Burkhadt and Bohm, 2007). Protocols to extract and quantify the carotenoid components of YP are more varied.

Current research indicates that carotenoids are the only known class of compounds that contribute substantially to YP in durum semolina. When comparing total carotenoids to total YP, correlation coefficients of 0.989, 0.980 and 0.94 were reported by Abdel-Aal et al. (2007), Fratianni et al. (2005) and Digesu et al. (2009), respectively. The major carotenoid is the xanthophyll *E*-lutein, reported to comprise about 80-90% of total carotenoids (Abdel-Aal et al., 2007). Including lutein fatty acid esters, the proportion of lutein has been reported to be as high as 99.9% (Lepage and Sims, 1968). Panfili et al. (2004) reported lutein (94%), zeaxanthin (4%) and α/β-carotene (3%) among the identifiable carotenoids in durum semolina. Hentschel et al.

(2002) and Burkhadt and Bohm (2008) found only lutein and traces of zeaxanthin, while Abdel-Aal et al. (2007) found that lutein comprised 86% of total carotenoids in semolina. An additional 5% was composed of *cis*(*Z*)-isomers of lutein; zeaxanthin accounted for 8% and the remainder was *β*-carotene. Fratianni et al. (2005) found that lutein comprised about 92% of the carotenoids in durum semolina; equal amounts of zeaxanthin and β-carotene accounted for most of the remainder. In einkorn wheat (*T. monococcum*), a correlation coefficient of 0.90 between lutein and total carotenoids was reported (Hidalgo et al., 2006). In summary, most of the minor carotenoids responsible for YP in semolina together comprise <20% of total carotenoids. These include zeaxanthin, esterified lutein, *Z*-isomers of lutein and zeaxanthin, and carotenes; most studies only report detecting some of these at any given time. This can be explained by the large variability in genetic backgrounds and environmental conditions during grain production, sample storage conditions, and varied extraction and HPLC techniques used among studies.

Besides the proportions of individual carotenoids, it is also useful to compare total carotenoids, usually identified and quantified by HPLC and/or mass spectrometry, to total YP. However, problems associated with this comparison may arise from a number of factors, such as poor recovery of carotenoids during extraction (Burkhadt and Bohm, 2007), incomplete identification of carotenoids (Panfili et al., 2004), comparison of dissimilar absorbance wavelengths and extinction coefficients between HPLC and YP assays (Abdel-Aal et al., 2007; Hentschel et al., 2002), differences in flour/meal particle size (Burkhadt and Bohm, 2007), and the presence of unknown non-carotenoid compounds that contribute to YP (Hentschel et al., 2002). All of these can potentially increase the discrepancy between the estimates of total carotenoids and YP, despite strong positive correlation. For example, Abdel-Aal et al. (2007) found that the YP assay overestimated the HPLC method by 20% in flours from a range of wheat species, while Hentschel et al. (2002) found an overestimation of 60% in durum semolina. However, Burkhadt and Bohm (2007) found virtually no difference between the two assays, with complete carotenoid recovery suggested to be the key factor. Similarly, Fratianni et al. (2005) found that the YP assay, on average, overestimated carotenoid

content by only 11%. To summarize, implementation of a standardized carotenoid assay for durum is desirable in order to compare results from different studies.

2.3 Carotenoids

2.3.1 Overview

Carotenoids are yellow, orange or red fat-soluble pigments (Moss and Weedon, 1976) that are present in photosynthetic organelles in plants, algae and cyanobacteria and serve an essential role by absorbing low wavelength light and preventing the degradation of chlorophyll (Cunningham and Gantt, 1998). Therefore, they can transfer absorbed light energy to chlorophyll and help in stabilizing chlorophyll complexes (Frank et al., 1997). They also react with oxidizers produced during photosynthesis, such as nascent oxygen and superoxide anions (Cunningham and Gantt, 1998). In addition, Their role as pigments in flowers is important in pollination (Cunningham and Gantt, 1998). In addition, they serve as precursors to other metabolites; the carotenoid zeaxanthin is a precursor to abscisic acid via the intermediates antheraxanthin, violaxanthin and xanthoxin (Cunningham and Gantt, 1998). Various carotenoids are also important in human nutrition for their antioxidant capacities and for their provitamin A activity (Cunningham and Gantt, 1998).

2.3.2 Chemistry

The basic structure of a carotenoid is a 40-carbon backbone composed of eight isoprene (C_5H_8) monomers, characterized by the presence of one or more light-absorbing chromophores when series of conjugated double bonds occur (Davies, 1976). The simplest carotenoid is phytoene ($C_{40}H_{64}$) (Cunningham and Gantt, 1998). Modifications to the basic acyclic structure, such as hydrogenation or dehydrogenation, cyclization, oxidation, epoxidation, isomerization, or any combination of these, give rise to the large diversity of these compounds in plants (Davies, 1976; Moss and Weedon, 1976). The structures of some common carotenoids are shown in Figure 1. As a group, they have absorption spectra occurring in the ultraviolet to visible region, usually characterized by the presence of triple maxima (Davies, 1976; Liaaen-Jensen and Lutnæs, 2008). These spectral peaks are often designated in the literature as I, II and III, with the strongest absorbance occurring at the middle wavelength (Chen et al, 2004;

Liaaen-Jensen and Lutnæs, 2008). In addition to absorbance, a distinguishing feature of a carotenoid spectrum is its fine structure, defined as the percentage ratio of its III/II absorbance peaks, using the intervening minimum as the baseline (Liaaen-Jensen and Lutnæs, 2008). Absorbance maxima generally increase with the number of conjugated double bonds (chromophores), but are also influenced by the presence of cyclic end-groups or chromophore side groups (Moss and Weedon, 1976).

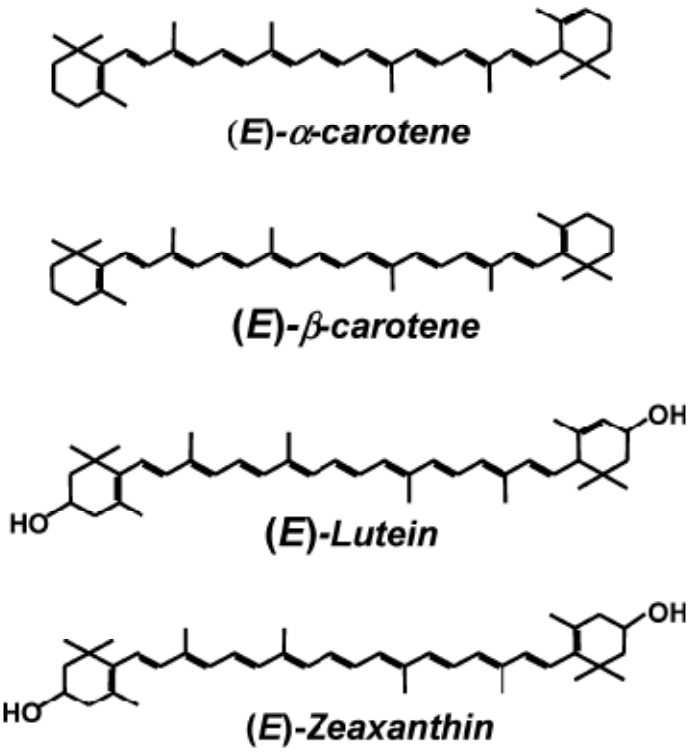

Figure 1. Structures of all-*E* forms of α-carotene, β-carotene, lutein and zeaxanthin, typical carotenoids found in plant tissues (Kean et al., 2007).

Carotenoids exhibit *Z/E* isomerism, with the number of possible Z stereoisomers depending on the number of non-cyclic double bonds, steric hindrance, and the symmetry of the carbon skeleton (Moss and Weedon, 1976). All-*E* carotenoids, which are the more stable and common form in plants, have the lowest solubilities, highest melting points and generally, the highest absorbance values. Isomerization to an equilibrium mixture of *E-Z* isomers can begin as soon as carotenoids are in solution; light, heat and catalysts accelerate it (Moss and Weedon, 1976; Liaaen-Jensen and

Lutnæs, 2008). Stereoisomers of carotenoids are often distinguished by the fact that mono-*Z* isomers absorb at wavelengths 2-5 nm less than that of the all-*E* isomer, known as a hypsochromic shift. The presence of two *Z* double bonds reduces the absorption wavelength maxima of carotenoids by about 10 nm (Moss and Weedon, 1976). The mono-*Z* isomers also have an additional spectral peak in the long UV region, approximately 150 nm below that of the wavelength of III; while poly-*Z* forms have highly reduced absorbances which occur at much shorter wavelengths (Liaaen-Jensen and Lutnæs, 2008; Moss and Weedon, 1976). The presence of the *Z* peak is used in the estimation of the Q ratio as described by Quackenbush (1987), which is also known as the A_b/A_{II} ratio (Britton, 1995). This is calculated as the ratio of the absorbance at the *Z* peak to the highest absorbance, which is generally at the middle wavelength (II). The highest Q ratios are exhibited by mono-*Z* carotenoids that possess the *Z* bond in the centre of the molecule (Liaaen-Jensen and Lutnæs, 2008). Aliphatic and monocyclic *Z* carotenoids exhibit two *Z* peaks (Liaaen-Jensen and Lutnæs, 2008). The *Z* carotenoids also tend to have reduced fine structure, which is defined as the ratio of the absorbance at the longest wavelength (III) to that of the middle wavelength (II), using the intervening minimum as the baseline (Britton, 1995). Fine structure decreases as the number of *Z* bonds increases, eventually approaching near zero in poly-*Z* isomers (Liaaen-Jensen and Lutnæs, 2008; Moss and Weedon, 1976). This is also observed in certain mono-*Z* isomers with hindered double bonds (Moss and Weedon, 1976). In the absence of NMR or MS data, the Q ratio, fine structure and hyposchromic shift are used in conjunction to assign tentative identifications to putative *Z*-isomers (Liaaen-Jensen and Lutnæs, 2008). However, this relies on the accurate identification of the related all-*E* isomer to use as a reference. Lastly, the stereoisomers have different adsorption affinities. This is the basis for their separation with chromatography (Moss and Weedon, 1976) and is discussed in section 2.3.7.

Carotenoids are degraded by acids and by oxygen and other oxidizing agents (Davies, 1976; Moss and Weedon, 1976; Saiz et al., 2001); a few are also degraded by alkali (Davies, 1976). Oxidizers such as benzoyl peroxide are used to eliminate carotenoids and associated yellow colour from bread wheat flour (Saiz et al., 2001). High-intensity light, particularly in the ultraviolet range, and heat also contribute to

carotenoid degradation (Davies, 1976). Siems et al. (1999) found that the rates of degradation (measured as loss of absorbance) upon treatment with sodium hypochlorite and exposure to UV light were both in the order lutein < zeaxanthin << lycopene < β-carotene. Exposure to direct sunlight had similar results. For example, 85% of lycopene and β-carotene was degraded after 36 h of sun irradiation, compared to 70% for lutein and zeaxanthin (Siems et al., 1999).

Carotenoids are broadly divided into two chemical classes: carotenes and xanthophylls. The latter are hydroxylated derivatives of carotenes (Kean et al, 2007) and are most soluble in moderately polar organic solvents, such as chloroform, cyclohexanone, diethyl ether, dimethyl sulfoxide, methyl-*tert* butyl ether and tetrahydrofuran, with moderate solubility in acetone, alcohols and acetonitrile (Craft and Soares, 1992). Carotenes are most soluble in non-polar or slightly polar organic solvents, such as alkanes, benzene, cyclohexane, dichloromethane and toluene, and have poor solubility in alcohols (Craft and Soares, 1992). Carotenoid polarities depend on the molecular structure and attached functional groups.

2.3.3 Biosynthesis in plants

The plant carotenoid biosynthetic pathway is shown in figure 2. Carotenoid biosynthesis begins with the condensation of two molecules of geranylgeranyl pyrophosphate (GGPP or GGDP) to form phytoene, catalyzed by the enzyme phytoene synthase (*Psy*) (Fig. 2). GGPP is derived from the isoprenoid precursor isopentenyl pyrophosphate (Cunningham and Gantt, 1998) and is also the precursor to the chlorophyll, phylloquinone, gibberellin and tocopherol pathways (Fraser et al., 2002). Phytoene is oxidized through four desaturation reactions that increase the number of conjugated double bonds from three in phytoene to eleven in lycopene (Moss and Weedon, 1976). The first two reactions are catalyzed by the enzyme phytoene desaturase (PDS), which produces the metabolic intermediates phytofluene and then ζ-carotene. The two subsequent reactions are catalyzed by ζ-carotene desaturase (ZDS), producing lycopene (Fig. 2). Lycopene cyclization then occurs, following one of two pathways that incorporate either the ε-ring or the β-ring in two-step reactions (Fig. 2). The asymmetrical carotenes formed after the first set of reactions undergo further

cyclization to form ε-carotene, α-carotene, or β-carotene (Cunningham and Gantt, 1998). The latter two are more common, although ε-carotene and its xanthophyll derivative, lactucaxanthin, occur in lettuce (*Lactuca sativa*) (Cunningham and Gantt, 1998). Carotenoid hydroxylase enzymes specific to the ε-ring and β-ring catalyze the double hydroxylation of α-carotene and β-carotene. This results in the formation of the xanthophylls lutein and zeaxanthin, respectively (Fig. 2). Zeaxanthin can undergo reversible double epoxidation of the rings, mediated by the enzyme zeaxanthin epoxidase (*ZEP*), to form violaxanthin, a precursor to abscisic acid. The interconversion of zeaxanthin to violaxanthin via the mono-epoxidated xanthophyll antheraxanthin is known as the xanthophyll cycle; the only major xanthophyll not part of this cycle is lutein (Cunningham and Gantt, 1998; Fig. 3). The primary carotenoid present in a given plant tissue depends on the organ and on the species.

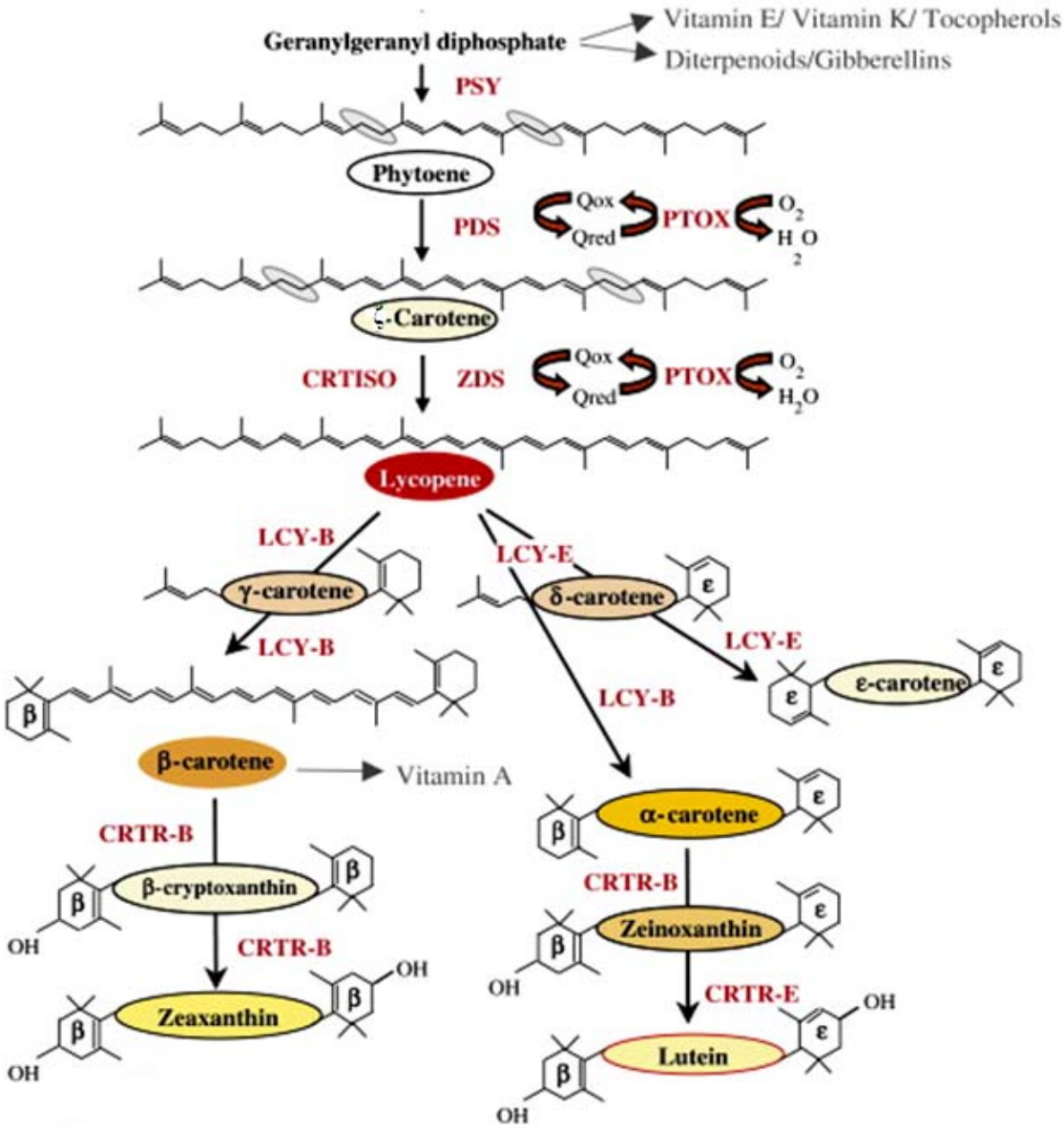

Figure 2. Carotenoid biosynthetic pathway (adapted from Pozniak, pers. comm.). PSY: Phytoene synthase; PDS: Phytoene desaturase; CRTISO: Carotenoid isomerase; ZDS: ζ-carotene desaturase; LYC-B: Lycopene-β-cyclase; LYC-E: Lycopene-ε-cyclase; CRTR-B: β-ring hydroxylase; CRTR-E: ε-ring hydroxylase.

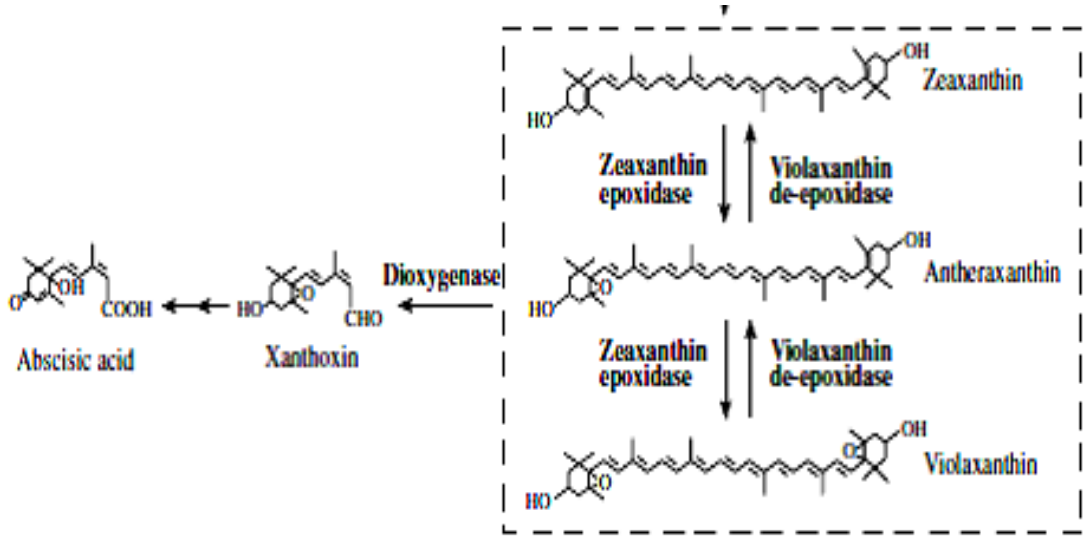

Figure 3. Zeaxanthin-violaxanthin pathway involving the enzyme *ZEP* (zeaxanthin epoxidase) and subsequent ABA formation (Cunningham and Gantt, 1998; cited by Yu et al., 2008).

2.3.4 Genetic control

2.3.4.1 In non-wheat species

The carotenoid pathway has been extensively characterized in several plant species, including members of the Poaceae. In addition to QTL-based mapping studies and analyses of allelic differences that change protein function, there have been numerous studies on profiling the transcript levels of carotenoid biosynthesis genes during development (Gallagher et al., 2004; Schofield and Paliyath, 2005; Clotault et al., 2008). The presence of multiple copies of *Psy* has also been reported in other grass species and was suggested to precede the evolution of this family (Gallagher et al., 2004). In maize (*Zea mays*), three copies of *Psy* have been reported, and two in rice (*Oryza sativa*) (Gallagher et al., 2004; Li et al., 2008). As in durum, Palaisa et al. (2003) found that allelic variation in *Psy1*, but not *Psy2*, was correlated to endosperm carotenoids in maize. In rice, white maize and yellow maize endosperm, *Psy2* was fully expressed and translated but *Psy1* was only expressed in yellow maize endosperm (Fig. 4) (Gallagher et al., 2004). Since *Psy2* also encoded a functional protein, it was suggested that the inability of this enzyme to localize to a plastid membrane resulted in the absence of carotenoid synthesis in rice and white maize. It was also suggested that

this duplication may be a mechanism to delink carotenoid synthesis in the grain from that in other parts of the plant (Gallagher et al., 2004). Similarly, Hirshberg (2001) suggested that the presence of separate, parallel chloroplast and chromoplast-specific pathways exists because of *Psy* and *CRTR-B* duplication. Evidence to support this was shown by Fray et al. (1993) where silencing of *Psy1* in tomato (*Lycopersicon esculentum*) affected fruit but not leaf carotenoid levels. Galpaz et al. (2006) showed that one paralog of the tomato *CRTR-B* gene (*CHY2*) was expressed only in flowers while the other paralog (*CHY1*) was only expressed in leaves. In Argentine canola (*Brassica napus*), maximal accumulation of lutein and β-carotene was detected at 35-40 days post-anthesis (DPA). However, transcript levels of *Psy*, *PDS*, *LCY-E* and *LCY-B* were highest between 20 and 30 DPA, coincident with maximal accumulation of violaxanthin (Yu et al., 2008).

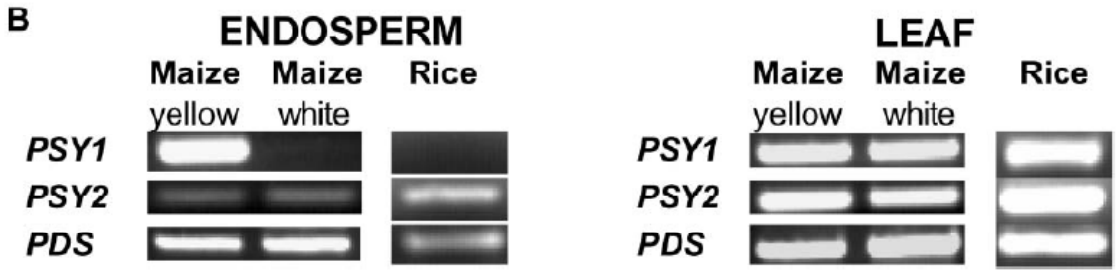

Figure 4. Transcript profile of phytoene synthase homeoforms (*Psy1* and *Psy2*) and phytoene desaturase (PDS) in maize and rice endosperm and leaves (Gallagher et al., 2004).

Similarly, Schofield and Paliyath (2005) noted an increase in phytoene synthase activity and the formation of phytoene with increasing transcript levels of *Psy* during tomato fruit ripening. In another study, *Psy* and *PDS* transcript levels were found to increase during tomato fruit ripening, coincident with lycopene accumulation, while *LCY-E* and *LCY-B* transcript levels decreased and eventually disappeared (Ronen et al., 1999). In *Arabidopsis thaliana*, *LCY-E* has been suggested to be crucial in regulating the ratio of lutein to β,β-xanthophylls, and mutants encoding less functional forms of *LCY-E* or *CRTR-E* have been observed to accumulate high levels of their substrates β-carotene and zeinoxanthin, respectively (Pogson et al., 1996).

To complement understanding of pathway regulation, there have been various studies on metabolically engineering carotenoid content in various plant species, either by enhancing the activity of genes controlling a rate-limiting step, introduction of a non-plant gene (Ye et al., 2000), or by gene silencing (Diretto et al., 2006). In a potato (*Solanum tuberosum*) cultivar with carotenoid concentrations similar to those of durum meal/semolina, its transformation with a functional *Erwinia uredovora* phytoene synthase gene (*crtB*) increased carotenoid content by up to 6-fold without any effect on ABA levels (Ducreux et al., 2005). Levels of lutein and particularly β-carotene were enhanced by this transformation, in terms of absolute concentration as well as proportion of total carotenoids; however, the levels of zeaxanthin, antheraxanthin, neoxanthin, violaxanthin and xanthophyll esters decreased in proportion. This was concurrent with a decrease in the transcript levels of *PDS*, as well as of *ZEP* and neoxanthin epoxidase (*NEP*) that are required for ABA synthesis (Ducreux et al., 2005). It was suggested that in transformed lines, the most significant rate limiting step was the activity of β-carotene hydroxylase (*CRTR-B*), resulting in a proportionate accumulation of its substrate and a decrease of its product, zeaxanthin (Ducreux et al., 2005). In another study on the same cultivar, silencing of *LCY-E* via antisense fragment insertion predictably increased the proportion of β,β-carotenoids relative to lutein, but because total carotenoid content also increased, the amount of lutein was comparable between transformed and control lines (Diretto et al., 2006). The transformation consistently induced increased expression of certain carotenoid pathway genes (*LCY-B*, *CrtISO* and *ZEP*), suggested by the authors to be by feedback mechanisms, but also possibly because of mutations occurring with the transformation (Diretto et al., 2006). A related study where *CRTR-B* paralogs (*CHY1* and *CHY2*) were silenced in the same cultivar resulted in a large accumulation of β-carotene and to a lesser extent, lutein and total carotenoids, in some transformed lines. Zeaxanthin levels decreased significantly but violaxanthin levels increased, possibly because of the induction of *ZEP*. Other genes induced were *ZDS*, *LCY-B*, *LCY-E*, and in one line, *CrtISO*, which could have been the reason for the increase in total carotenoids (Diretto et al., 2007). In both gene silencing studies, a moderate increase in phytofluene content was observed. However, this product is far upstream of both *LCY-E* and *CRTR-B*, as it is an intermediate in the

formation of ζ-carotene from phytoene. Both results were noteworthy because partial suppression of a downstream enzyme not only changed the individual carotenoid proportions but also increased the overall carotenoid content. It was suggested that the accumulation of any of the reported carotenoids could have induced several genes in the pathway, thereby increasing total carotenoid levels through a self-regulatory mechanism.

In *B. napus* seeds, silencing of *LCY-E* with RNA*i* reduced the level of the corresponding transcript as well as that of *Psy*, without affecting transcript levels of *PDS* and *LCY-B* (Yu et al., 2008). Paradoxically, transgenic lines contained significantly higher amounts of carotenoids, including lutein. An increase in ABA activity was also suspected, although this was not directly measured. As expected, β-carotene levels were enhanced the most, producing a higher ratio of β-carotene to lutein in transgenic lines. The levels of β,β-xanthophylls also increased slightly. Although an increase in β,β-carotenoids in transgenic lines was anticipated if *LCY-E* was rate limiting, the increase in lutein was not. To explain this, it was suggested that compensation from a homolog of *LCY-E*, or compensation from a different enzyme with a broad substrate range, or an increase in the efficiency of lutein compartmentalization may have been responsible (Yu et al., 2008).

E. uredovora has also been used in transforming other species for novel carotenogenesis. Ye et al. (2000) used its *PDS* (*crtI*) with a daffodil *Psy* to transform rice to enable β-carotene production in the endosperm. In canola (*Brassica napus*), a fifty-fold increase in total seed carotenoids occurred after targeted overexpression of *E. uredovora Psy (crtB)* (Shewmaker et al., 1999). Fraser et al. (2002) observed a 60% increase in total carotenoids after overexpression of the same construct in tomato. In this study, the amount of *Psy* protein increased exponentially with lycopene synthesis in the later stages of fruit ripening.

2.3.4.2 In wheat (Triticum spp.)

Research into the mechanisms regulating carotenoid synthesis in durum is relatively recent and has focused on genes in the biosynthesis pathway. A QTL for YP on chromosome 7B (section 2.2.2) was identified as *Psy-B1*, a gene coding for phytoene

synthase (Fig. 2) (Pozniak et al., 2007); the sequence difference between the high-YP and medium-YP parents was in putative introns. Its homeolog on 7A (*Psy-A1*) was also identified as being associated with YP in a second durum population (Singh et al., 2009) and in bread wheat (He et al., 2008). In the latter study, this was explained by an insertion in the 5' end of the second intron and a SNP in the fourth intron, leading to reduced YP. It was hypothesized that this sequence difference led to an alternative splicing event that prematurely terminated translation, and consequently, protein function. The same study also found high similarities between the wheat *Psy-A1* and maize *Psy1* sequences, indicating that the gene sequence is highly conserved. Similar results were reported by Howitt et al. (2009) in bread wheat, where alternative splicing in the low-YP *Psy-A1* allele reduced the amount of wild-type mRNA, and consequently protein function. Zhang and Dubcovsky (2008) reported that a mutant sequence of *Psy1* in tall wheatgrass (*Lophopyrum ponticum*) in recombinant durum lines was associated with reduced YP. They also reported association of an allele, *Psy-A1c* (Reimer, 2008) with low YP. Subsequently, this allele was renamed *Psy-A1a* (Singh et al., 2009). Singh et al. (2009) reported a significant association between allelic variation in *Psy-A1* and durum grain YP. Although most of the sequence variation was in introns, there also existed a sequence difference in a region coding for the chloroplast transit peptide of the gene. This differentiated an allele (*Psy-A1o*), associated with high-YP, from the two other alleles, *Psy-A1a* and *Psy-A1l*. Allelic variation at *Psy-B1* and *Psy-A1* that has been associated with YP was also reviewed by Reimer (2008).

Unpublished work suggests that *LCY-B* (Fig. 2) is responsible for a QTL for YP on 6B (Pozniak, personal communication, 2009). It is also possible that the QTLs for YP reported on the group 2 and group 4 chromosomes may be explained by *ZDS* and *PDS* (Fig. 2) respectively, as they map to these chromosomes (Cenci et al., 2004). Sequence variation in the coding region of *LCY-E* has been suggested to regulate lutein and total carotenoid content in hexaploid wheat because of a single nucleotide polymorphism that resulted in an amino acid substitution. This substitution was suggested to have enhanced *LCY-E* activity, increasing lutein content relative to the wild-type cultivar (Howitt et al., 2009).

Gene duplication of *Psy* has also been reported, although the extent to which the copies regulate the carotenoid pathway is unknown. It is also hypothesized that other genes in the pathway may be duplicated (Gallagher et al., 2004). Pozniak et al. (2007) reported a second pair of *Psy* genes (*Psy-2*) on the group 5 chromosomes. Despite allelic variation, no association with YP was reported. Singh et al. (2009) suggested that the presence of a third *Psy* copy in durum could not be ruled out because it could be responsible for the presence of an addition QTL associated with YP on chromosome 7A. Gene duplication has also been reported in hexaploid wheat (Gallagher et al., 2004).

To summarize, the mechanisms behind how carotenoid products regulate genes upstream or downstream in the pathway are still largely unknown. Although naturally high-carotenoid species are less easily transformed to accumulate more carotenoids (Fraser et al., 2002), perturbations of carotenoid profiles often create unexpected feedback effects. The presence of carotenoid gene homologs, homeologs and paralogs in polyploid plants may be a challenge when breeding for altered carotenoid content or profiles.

2.3.5 Distribution in the wheat kernel

Although wheat caryopses contain various antioxidant phytochemicals such as polyphenols and flavonoids, they are predominantly found in the bran and germ (Adom et al., 2005) and are lost during conventional processing. Carotenoids are found throughout the kernel in durum and in related species, including einkorn and bread wheat (Adom et al., 2005; Hentschel et al., 2002; Hidalgo and Brandolini, 2008). They are compartmentalized in amyloplasts, which are modified plastids for starch storage (Howitt et al., 2006). Hentschel et al. (2002) found that in durum, the endosperm had the highest lutein and total carotenoid concentration, although total YP concentrations were generally highest in the bran and germ. Fratianni et al. (2005), upon examining a range of low-YP durum wheat cultivars, reported approximately 15% more lutein, zeaxanthin and β-carotene in whole meal than in semolina. There were no differences in particle sizes between the two fractions, as particle size can influence carotenoid extraction recovery. On the contrary, Abdel-Aal et al. (2007) found a 30% higher concentration of lutein in the endosperm of durum than in the bran. Similar patterns were seen in other

wheat species (einkorn and Khorasan), the only exception being bread wheat that had about 30% higher lutein concentration in the bran. Indeed, Adom et al. (2005) found that the bran and germ of bread wheat had over three times the level of total carotenoids (lutein, zeaxanthin and β-cryptoxanthin) as the endosperm. These studies suggest that the distribution of carotenoids in the kernel strongly depends on whether the genotype is a high-YP or low-YP cultivar or species. As the germ is higher in fatty acids, LOX activity will also be higher in whole meals.

2.3.6 Health benefits

Carotenoids may have numerous advantages for human health. In addition to the provitamin A activity of certain carotenoids, primarily β-carotene and to lesser extent α-carotene and β-cryptoxanthin (Davey et al., 2009), they may help in preventing age-related macular degeneration (AMD) by protecting the fatty acid-rich outer layer of the retina from free radical oxidative damage (Seddon et al., 1994). This antioxidant benefit has been suggested in several animal studies reviewed by Seddon et al. (1994). They may also protect the eye by absorbing excess blue light, thus preventing light damage to the retina (Seddon et al., 1994). Their study in 876 human subjects suggested a 43% lower risk of AMD in people with the highest consumption of carotenoids (median intake of 19 mg/day) than in those with the lowest intake (median of 3 mg/day). Based on this study, significant reductions in the risk of contracting AMD could be observed with a total carotenoid intake ≥ 5 mg/day. There was a significant and linear negative trend between increasing carotenoid intake and the development of AMD, and the strength of this relationship increased when only xanthophylls (lutein and zeaxanthin) were considered in the model (Seddon et al., 1994). These xanthophylls dominate the carotenoid profile in the macula of the human retina (Seddon et al., 1994). The intake of β-carotene was also AMD-preventative, albeit less so than lutein and zeaxanthin (Seddon et al., 1994). Among common carotenoids, lycopene was the only one that had no significant effect on AMD (reviewed by Seddon et al., 1994). Olmedilla et al. (2001) reported that cataract patients receiving lutein supplementation of about 6.5 mg/day showed significant improvements in visual acuity within six months. Therefore, carotenoid intake may not only prevent AMD but also partially reverse it. The antioxidant activity of carotenoids may also have an anti-carcinogenic effect (Krinsky,

1989). Kabat et al. (2009) found an inverse correlation between serum levels of α-carotene and β-cryptoxanthin and breast cancer. Similarly, Mignone et al. (2009) found a strong and significant inverse relationship between dietary consumption of carotenoids (carotenes as well as xanthophylls) and breast cancer; this protective effect was even stronger in smokers. In mice (*Mus musculus*), zeaxanthin supplementation inhibited lung cancer by 71% and liver cancer by 93% (Nishino et al., 2009). Other carotenoids had similar effects; lutein suppressed skin and colon cancers, and carotenes (particularly α-carotene) suppressed the development of a range of cancers (Nishino et al., 2009). A combination of various carotenoid supplements was the most effective in anti-carcinogenic activity; this was also observed in humans in a previous study (Le Marchand et al., 1993). Lycopene and β-carotene, but not lutein, were also found to increase the rate of DNA repair in human lymphocytes after oxidative stress (Torbergsen et al., 2000). Nevertheless, not all studies have found a significant benefit from increased carotenoid intake (reviewed by Mozaffarieh et al., 2003); therefore, their role in human health has not been conclusively established, nor is there a maximum recommended intake. A list of the ranges in carotenoid concentrations typically found in some food products is given in Table 1. In addition, the bioavailability of carotenoids must be considered because lutein absorption from plant matrices tends to be relatively low. Chung et al. (2004) found that lutein absorption was higher from eggs than from spinach or commercial supplements, despite sufficient fat to enable absorption. Using supplements as a control, Van het Hof et al. (1999) found that lutein uptake was 30% lower from mixed vegetables, and Castenmiller et al. (1999) found that it was about 50% less from spinach. However, when consumed with a larger amount of fat (55-60% of food energy), there were no significant differences (Chung et al., 2004).

Among common antioxidant phytochemicals present in food, carotenoids tend to have reasonably high cooking and processing stabilities; their stability relative to other phytochemicals is greater under increasingly destructive cooking treatments. They are significantly more resistant to cooking or processing loss than ascorbic acid, polyphenols and anthocyanins (Chuah et al., 2008; Parra et al., 2007) but less so than tocopherols (Hidalgo et al., 2008).

Table 1. Approximate carotenoid concentrations in selected raw and cooked food items (dry matter basis unless indicated as f.w.)

Food Category	Food item	Total Carotenoids (µg g $^{-1}$)	Xanthophylls (µg g $^{-1}$)	Sources
Cereal Products	Bread wheat (flour and meal)	0 - 3	0 - 3	Abdel-Aal et al. (2007), Hidalgo and Brandolini (2008), Howitt et al. (2009), Humphries and Khachik (2003), Liu (2007)
	Durum (semolina)	2 - 7	2 - 7	Abdel-Aal et al. (2007), Burkhadt and Bohm (2008), Fratianni et al (2005), Hentschel et al. (2002), Panfili et al. (2004)
	Einkorn (flour and meal)	4 - 13	4 - 13	Abdel-Aal et al. (2007), Hidalgo et al. (2006), Hidalgo and Brandolini (2008)
	Maize, yellow (endosperm)	10 - 35	8 - 34	Abdel-Aal et al. (2007), Burkhadt and Bohm (2008), Kean et al. (2008), Kimura et al. (2007)
	Maize, yellow (bran)	2 - 7	1 - 5	Kean et al. (2008)
	Maize, yellow (whole meal)	8	7	Parra et al. (2007)
	Maize, yellow (nixtamalized whole meal)	3	3	Parra et al. (2007)
	Pastry wheat (meal)	1 - 2	1 - 2	Roose et al. (2009)
	White bread, bleached (f.w)	0.2	0.2	Perry et al. (2009)
Fruits and Vegetables	Asparagus	100 - 115	78 - 86	Tenorio et al. (2004)
	Carrot (f.w)	20 - 110	2 - 4	Clotault et al. (2008)
	Kale (cooked, f.w)	60 - 144	37 - 89	Perry et al. (2009), Sa et al. (2003)
	Mango	60	20	Chen et al. (2004)
	Radish leaves	430	320	Lakshminarayana et al. (2005)
	Red pepper (f.w)	53	-	Chuah et al. (2008)
	Red pepper (boiled, f.w).	43	-	Chuah et al. (2008)
	Spinach	2020	2020	Lakshminarayana et al. (2005)
	Spinach (f.w)	123	72	Perry et al. (2009)
	Spinach (fried, f.w)	236	135	Perry et al. (2009)
	Tomato (f.w)	9	Trace	Perry et al. (2009)
	Egg (raw, f.w)	7	7	Perry et al. (2009)
Other	Egg (cooked, f.w)	6	6	Perry et al. (2009)
	Egg, high lutein (cooked, f.w.)	30	30	Chung et al. (2004)
	Olive oil (f.w)	3 - 13	2- 10	Minguez-Mosquera et al. (1992)
	Pesto sauce (f.w)	180 -500	80 - 230	Masino et al. (2008)

For example, about 75% of the lutein in einkorn meal samples was retained after high temperature (120°C) and pressure (2.1 bar) cooking for 15 minutes (Hidalgo et al., 2008). In paprika and bell pepper (*Capsicum annuum*), 60-80% of total carotenoids, mostly xanthophylls (Perry et al., 2009), were retained after boiling for 30 minutes; microwaving and stir-frying enabled even higher recoveries (Chuah et al., 2008). Lutein, and to a smaller extent zeaxanthin, have greater cooking stability than other carotenoids commonly present in food items, such as β-carotene and β-cryptoxanthin (Parra et al., 2007; Perry et al., 2009). Given that lutein is the major carotenoid in durum, it implies that relatively high YP and antioxidant value can be expected to persist in cooked pasta if it is processed appropriately. Based on the data in Table 1, and the results of the epidemiological studies, one 100 g serving of pasta a day would provide about 10% of the minimum intake (5 mg) for xanthophylls, assuming no processing loss and complete absorption. This indicates that semolina and pasta may be good sources of xanthophylls (Health Canada, 2005). The relatively low fiber content of pasta, compared to vegetables, may be beneficial in carotenoid absorption. Nevertheless, as there is no officially established recommended dose for lutein or other xanthophylls, high-YP pasta can only be one of several foods relied upon to collectively supply a therapeutic dose.

2.3.7 Analysis

2.3.7.1 Identification and Extraction

Carotenoids can be identified and structurally examined by various physical or chemical methods, or a combination of them (Moss and Weedon, 1976). Electronic spectroscopy (ES, also known as UV-visible spectroscopy or diode array detection), utilizing differences in absorption spectra, is the most common method. Mass spectrometry (MS) is a more accurate technique that can be used to confirm the identity and structure of carotenoids, but it is sensitive to impurities and may not be appropriate for all types of carotenoids (Maoka, 2009). For example, Abdel-Aal et al. (2007) used positive electrospray ionization MS to confirm carotenoid identification in durum. This technique was also used by Maoka (2009) to elucidate the molecular formula of a carotenoid in *Celastrus orbiculatus*. Mercadante et al. (1998) used electron impact MS to identify carotenoids in passion fruit (*Passiflora edulis*), while Rehbein et al. (2007)

used atmospheric pressure chemical ionization MS to confirm the identity of the carotenoid bixin. Nuclear magnetic resonance (NMR) spectroscopy and circular dichroism spectrometry (CD) are also occasionally used in the structural analysis of carotenoids, particularly minor carotenoids (Maoka, 2009). The advantages of NMR and CD spectrometry, particularly for unstable carotenoids, are that they can be used with minimal method development and without potentially destructive extraction steps (Valverde and This, 2008; Zsila et al., 2001). For example, in Valverde and This (2008), lutein and β-carotene were extracted with acetone, partitioned into cyclohexane, evaporated and redissolved before being identified and quantified using two-dimensional proton-correlation NMR (^{1}H-^{1}H-COSY). NMR is also useful in differentiating between stereoisomers of carotenoids (Rehbein et al., 2007). Maoka (2009) used proton (δ 4.00) and C13 (δ 65.1) spectra to assign an identity, *Z*-celaxanthin, to the carotenoid found by MS. Rehbein et al. (2007) used a 14.1 T cryomagnet in a carotenoid NMR spectroscopic technique similar to that used by Valverde and This (2008). Zsila et al. (2001) used a CD spectropolarimeter (>300 nm) to help elucidate the optical activity of carotenoids in flower petals. Infrared spectroscopy and chemical reaction-based techniques are used less commonly (Moss and Weedon, 1976). X-ray crystallography can be used for confirming the molecule's absolute configuration, although in practice it has technical limitations in carotenoid research (Moss and Weedon, 1976) and has not been used to the extent of other methods. Chemical methods of identification are used less often, but may be useful in confirming the presence of specific carotenoids (Moss and Weedon, 1976). The most common methods are based on hydride reduction, acid and base-induced modification or degradation, and oxidation (Moss and Weedon, 1976).

Protocols for the extraction of carotenoids from a biological matrix vary depending on carotenoid profiles, quantification methods and technical considerations. For maximum carotenoid recovery, samples are usually frozen or freeze-dried, with or without liquid nitrogen (Davies, 1976; Esteban et al., 2009). Samples may also be desiccated in silica gel or stored in acetone for short periods prior to extraction (Esteban et al., 2009). Samples suspected to contain a significant amount of esterified carotenoids may be submitted to a pre-extraction saponification step with alcoholic KOH or NaOH

(Davies, 1976; Panfili et al., 2004). This also removes chlorophylls and their products which may interfere with carotenoid analysis (Davies, 1976; Shi and Chen, 1999). Antioxidants such as butylated hydroxytoluene (BHT) or butylated hydroxyanisole (BHA) (Hentschel et al., 2002), or pyrogallol (Panfili et al., 2004), are typically included and extractions may be conducted in dim light or darkness (Davies, 1976; Abdel-Aal et al., 2007) and/or under nitrogen (Moss and Weedon, 1976) to minimize carotenoid degradation. Magnesium or calcium carbonate may be included to prevent acid-induced carotenoid degradation (Esteban et al., 2009; Hentschel et al., 2002; Valverde and This, 2008). Typical extraction procedures adopted for LC-MS analysis are summarized in Figure 5.

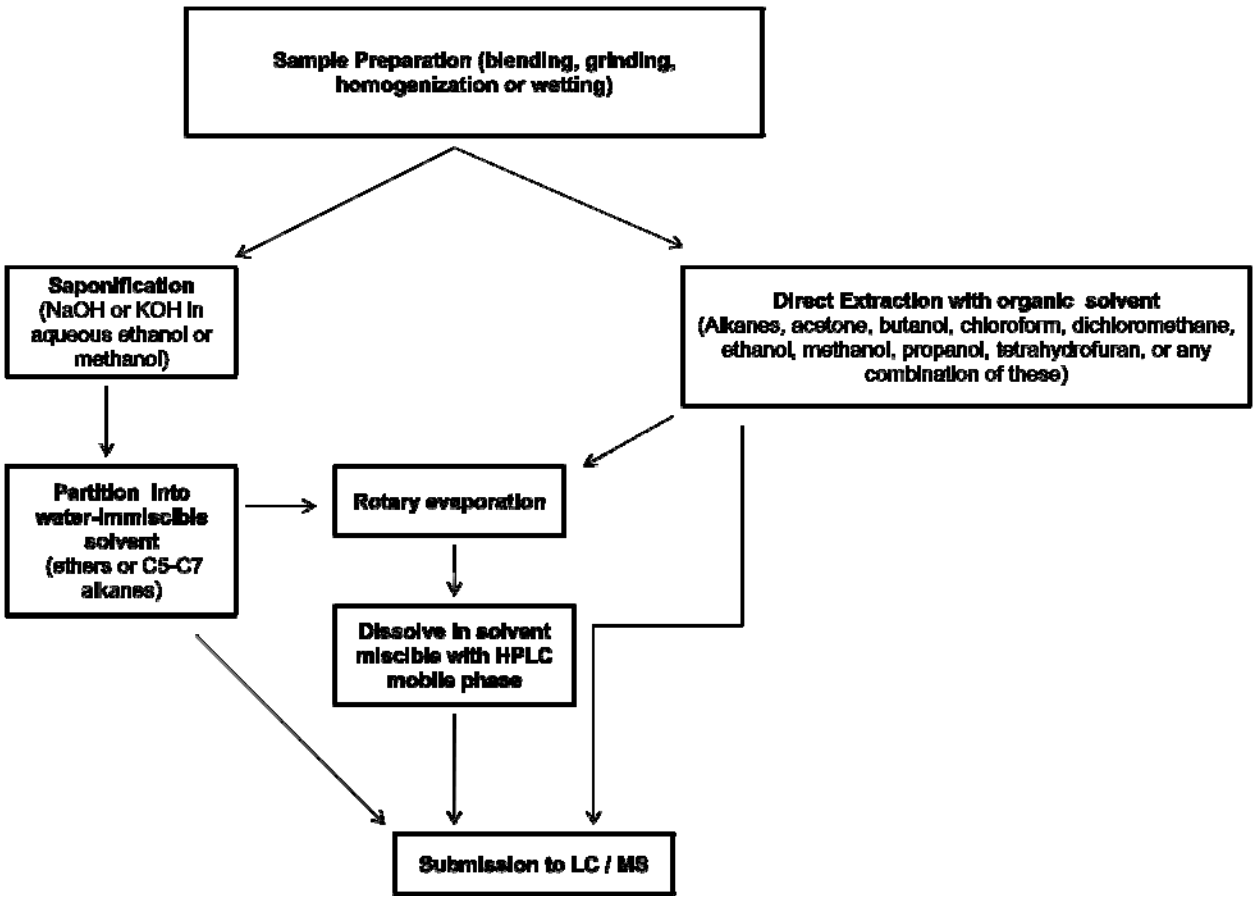

Figure 5. Overview of common carotenoid extraction protocols for HPLC/MS analysis

2.3.7.2 Separation

Carotenoid separation is also accomplished through diverse means. The simplest method is phase separation. Carotenes, monohydroxycarotenoids and dihydroxycarotenoids (xanthophylls) can be separated during extraction by using alkanes/ether, 95% methanol and 90% methanol, respectively (Davies, 1976). However, with the advent of chromatographic separation techniques, this procedure is uncommon except as a preparatory technique. Paper chromatography, thin layer chromatography (TLC) and column chromatography utilize an adsorbent surface to separate carotenoids based on their adsorption affinities (Davies, 1976). Although the first two methods are relatively poor in performance, they remain popular because of their low cost and ease (Scott, 1996). Three major types of column chromatography exist: zone chromatography, stepwise elution chromatography, and gradient elution chromatography. Gradient elution is the most modern of these methods and is the principle behind high-performance liquid chromatography (HPLC) (Davies, 1976). Carotenoid analysis is derived from a coupling of a separation method with a suitable identification method, such as LC-MS,LC-DAD or LC-NMR (Rehbein et al., 2007).

2.3.7.3 Overview of High Performance Liquid Chromatography (HPLC)

Chromatography, as defined by Meyer (2004), is a type of separation process where a mixture of sample components is distributed between an active stationary phase (usually porous solid particles, or a solid wall coated with liquid) and a fluidic mobile phase. However, some variants exist. For example, Luo et al. (2003) used a chromatographic system where liquid methanol saturated with carbon dioxide acted as the stationary phase, and the gaseous mobile phase was composed of carbon dioxide saturated with methanol vapour. With the exception of gas chromatography (GC), mobile phases are liquids. HPLC is also known as high pressure liquid chromatography (Meyer, 2004); the need for a high pressure pump arose with the use of separation columns (stationary phases) tightly packed with small particles that required a controllable flow rate (Ardrey, 2003; Scott, 1996). Therefore, unlike other, older types of LC, HPLC is characterized by its speed, efficiency, reproducibility and accuracy (Ardrey, 2003; Meyer, 2004). HPLC has advantages over GC for analysis of non-volatile or thermally labile compounds, while its main drawback over other analytical

techniques is its inability to analyze insoluble compounds (Meyer, 2004). Separated compounds are then analyzed by an appropriate detection method. A typical HPLC system is represented in Fig. 6.

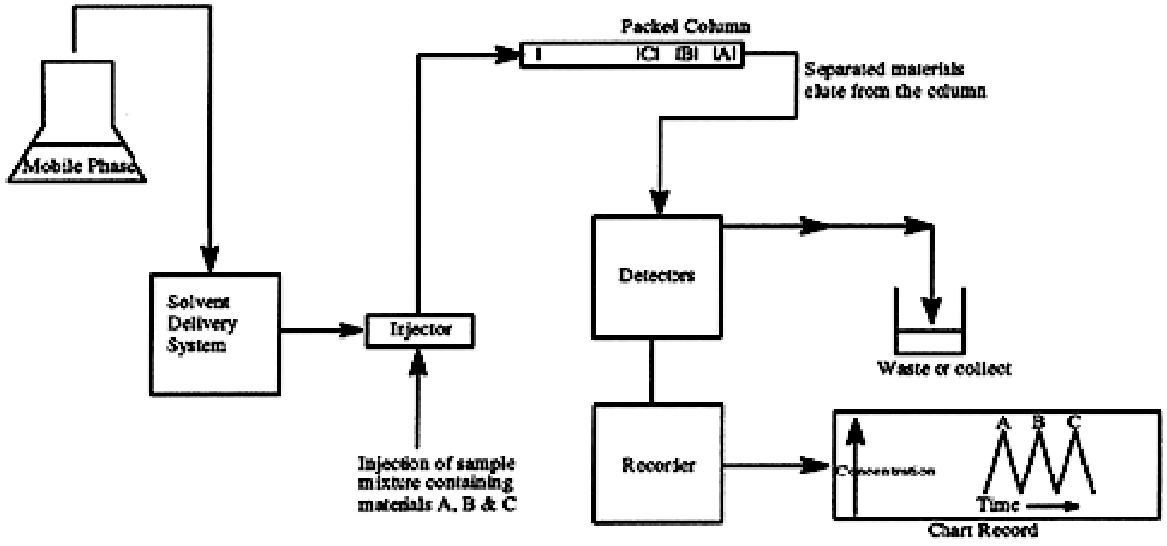

Figure 6. Schematic representation of a typical HPLC system. (**http://www.protein.iastate.edu/hplc.html**)

2.3.7.4 Types of HPLC separation methods

Broadly, HPLC methods can be grouped into four chromatography classes on the basis of separation: partition, size exclusion, ion exchange and affinity (McMaster, 2007).

Partition chromatography (PC), which is the most common HPLC method for carotenoid analysis, is based on the principle that sample components in the mobile phase will separate upon interacting chemically with the stationary phase. Components of similar polarities attract each other; therefore, they equilibrate between the stationary and mobile phases. As a result, components with the most affinity to the stationary phase elute last (McMaster, 2007). This method can divided into two types – normal phase (NP) chromatography (also known as adsorption chromatography), and reversed phase (RP) chromatography (Meyer, 2004). The former, which derives its name from conventional TLC and column chromatography, consists of a relatively polar stationary phase (column), usually silica, or occasionally alumina or magnesium oxide. The

polarity of the stationary phase is derived from hydroxyl groups of silicic acid or from aluminum ions in silica and alumina columns, respectively (Meyer, 2004; Scott, 1996). Particles of this stationary phase enable a large surface area, and the mobile phase is relatively non-polar. The opposite is true for RP chromatography, where the column is coated with a highly non-polar chemically bonded compound as the stationary phase (Meyer, 2004). Organic polymel gels may also be used as a stationary phase (Svec, 2004). This bonded phase is often hydrocarbon-based (Scott, 1996), with a carbon chain length of one to eighteen (Arakawa and Philo, 2007) or even 30. Hydrophobic components of sample compounds interact more strongly with the stationary phase (Arakawa and Philo, 2007) than with the polar aqueous or alcohol solvent systems that characterize the mobile phase.

Ion exchange chromatography (IEC) is based on ionic interactions between sample components and the stationary phase. As in RP chromatography, columns are typically silica or zirconium or cross-linked organic polymers, which in turn are bonded to organic phases with charged functional groups (McMaster, 2007). The strength of attraction between them is directly proportional to elution time. This method is useful for separating compounds with ionic groups, such as amino acids and organic acids (Meyer, 2004). Size exclusion chromatography (SEC) is used to separate components based on differences in molecular size, which is inversely proportional to elution time (McMaster, 2007). This is often used industrially for fractionating mixtures, desalting proteins, or removing ethanol from aqueous samples (Kale and Cheryan, 2009). Affinity chromatography (AC), which is the least common of the separation methods, is characterized by strong specificity exhibited by the column towards a class or group of sample components (McMaster, 2007). This method may often be used for protein, lipid or immunological analysis (Meyer, 2004).

2.3.7.5 HPLC Detectors

For some detectors, accurate quantitative detection is limited to the 'linear range', where the chosen property of the solute/component is directly proportional to its concentration or mass. The dynamic range includes the levels of solute that are beginning to saturate the detector, while the detection threshold marks the lower limit of

the linear range (Ardrey, 2003). HPLC detectors can be classified in several ways: i) solute or solvent property detectors; ii) general or selective detectors; iii) mass or concentration-based detectors (Ardrey, 2003). The four most common detectors are UV-Vis detectors, fluorescence detectors, conductivity detectors and refractive index detectors (Scott, 1996). Evaporative light scattering (ELS) detectors are also occasionally used, such as for carbohydrate analysis (Kabel et al., 2003).

UV-Vis detectors, used for detecting compounds that absorb in the UV and visible light range, fall into three subtypes: fixed wavelength, variable wavelength, and photodiode array (PDA). The PDA detector is the most versatile and modern of these (Scott, 1996). Fluorescence detectors, although highly sensitive, are used for a relatively narrow range of compounds that fluoresce at a given wavelength that can be emitted by the detector (Scott, 1996). Conductivity detectors are used for ionic sample compounds, such as salts, bases and acids, including amino acids and organic acids (Scott, 1996). Refractive index detectors are less common and are used when other detectors are inappropriate, such as for certain carbohydrates, alcohols and aliphatic compounds. These detectors cannot be used with the gradient elution that is common in HPLC (Scott, 1996). Additionally, separated compounds can be submitted to an external mass spectrometer (MS) via an interface, such as an in-line or Z-spray electrospray interface (Ardrey, 2003), to support HPLC detector results, or to identify unknown compounds. Identification based on MS spectra carries a higher degree of confidence than that based on UV-Vis absorption spectra (Ardrey, 2003). Briefly, mass spectrometry is a technique where sample components are 'softly' ionized through various methods, including electron ionization, electrospray ionization, matrix-assisted laser desorption ionization, or fast atom bombardment. These ions are routed through a vacuum to reach the detector, which separates and identifies them based on their mass to charge ratio (Ardrey, 2003).

2.3.7.6 HPLC for carotenoid analysis

The use of SEC, IEC and AC for direct carotenoid separation is limited, although they may be useful in preparation or purification protocols, or as complementary analytical tools. For example, Kale and Cheryan (2009) used SEC to separate corn zein

from xanthophylls. Although it was not performed here, the eluted xanthophylls could be submitted to PC for separating individual components or as a source for synthesizing standards. IEC was used to separate lutein, zeaxanthin and xanthophyll-binding proteins of the human retina (Yemelyanov et al., 2001). Similarly, Bhosale et al. (2009) used IEC followed by SEC to purify a lutein-binding retinal protein.

Both NP and RP chromatography (C18 and C30 columns) have been used to separate carotenoids and their stereoisomers, based on the differences in carotenoid polarities. The main difference between them is the reversal of elution profiles. Until recently, NP columns were better at separating lutein, zeaxanthin and their isomers than RP columns, but not carotenes (Goodwin and Britton, 1988; Panfili et al., 2004). However, the use of NP protocols decreased as these columns were less reliable or flexible and had long equilibration times, despite their superior separation of polar isomers (Meyer, 1997). They were also not compatible with the more aqueous mobile phases that would be used for more polar biological compounds, nor were they compatible with solvent gradients of varying polarities (Meyer, 1997). The introduction of long chain C30 columns also improved RP separation of carotenoid isomers, making it the most popular HPLC method for carotenoid analysis. Nevertheless, NP columns can be used if mobile phase solvents are within a narrow polarity range (C_5-C_7 alkanes to dichloromethane) (Meyer, 1997), as was demonstrated by Humphries and Khachik (2003), Fratianni et al. (2005) and Panfili (2004). Instead, mobile phases for RP columns typically include a gradient to improve baseline separation, and a relatively non-polar solvent may be included to modify polarity. Gradients are typically composed of methanol/dichloromethane (Hu et al., 2007), methanol/methyl *tert*-butyl ether (Abdel-Aal et al., 2007), methanol/acetonitrile (Hentschel et al., 2002), and often an optimized mixture of methanol, acetonitrile and dichloromethane (Lakshminarayana et al., 2005; Goodwin and Britton, 1988). A gradient of ethyl acetate (0-100%) in acetonitrile/methanol and water has also been used (Goodwin and Britton, 1988; Norris et al., 1995; Pogson et al., 1996). Carotenoids are detected by UV-vis spectroscopy, with absorption maxima ranging from 275 nm for phytoene to over 500 nm for lycopene (Tan et al., 1988). MS-based detection may be used to confirm identification.

Fluorescence detection is rarely employed because of the extremely low quantum yields (10^{-4} to 10^{-5}) (Frank et al., 1997).

2.4 Thesis Objectives and Hypothesis

Most research to date has focused on YP as a phenotypic marker for carotenoids, but this ignores differences amongst individual carotenoids that could be valuable in mapping or association studies. Little is known about the regulation of carotenoid biosynthesis in durum, although the carotenoid biosynthetic pathway has been characterized in several related species, including maize and bread wheat (Gallagher et al., 2004, He et al., 2007). The objective of this research thesis is to indicate whencarotenoid biosynthesis and accumulation occur in durum wheat and what their relationship is with measured YP values. It is also necessary to obtainclues on the mechanisms of regulation. Based on existing genetic and physiological data, we hypothesize that the differences in YP content among genotypes are influenced by carotenoid content, especially lutein. It is also hypothesized that these differences may involve either rate or time of accumulation and/or degradation of carotenoids at different stages of grain development. To achieve these objectives and to test the hypotheses, it is first necessary to understand carotenoid accumulation patterns through grain fill in durum wheat cultivars and breeding lines with varying expression of endosperm YP. This could provide clues as to rate-limiting genes in the carotenoid biosynthetic pathway which could then be the target of more detailed genetic studies. Based on existing QTL and phenotypic data, it can be hypothesized one or more genes, including *Psy1*, may influence carotenoid accumulation patterns (and therefore YP) because of genetic variation amongst genotypes. In addition to the rates and periods of accumulation, the ratios of certain carotenoids and the proportion of YP represented may indicate what genes are regulatory or rate-limiting.

3. MATERIALS AND METHODS

3.1 Plant Material

Five durum cultivars and eight breeding lines with a wide variation in YP concentration were included in this study. The cultivars were Commander (Clarke et al. 2005b) and Strongfield (Clarke et al. 2005a) from Canada, Demetra from Italy, Novadur from France and Kofa from the USA. The breeding lines comprised 2805 from Germany, 940435 from Australia, IDYT-020 (denoted here as A0600B*020) from the ICARDA 2006 International Durum Yield Trial (IDYT) for dry continental areas, and the remainder (A9831-DC*1, A0022&D509, DT696, DT707 and W9262-260D3) from the Agriculture and Agri-Food Canada durum breeding program at Swift Current, SK. Kofa and W9262-260D3, and Strongfield and Commander are parents of two independent mapping populations used to identify QTL associated with YP (Pozniak et al. 2007; Singh et al. 2009). The 13 genotypes were grown in 4.5 m^2 plots in a randomized complete block design at the Kernen Crop Science Research Farm, Saskatoon, SK ('Kernen'; three replicates) and in 2.74 m^2 plots at Agriculture and Agri-Food Canada, Swift Current, SK, Canada ('SC'; two replicates), in 2007 and 2008. Developing spikes were sampled at 10, 14, 21, 28 and 35 days after heading (DAH) and stored at -20°C. The rest of each plot was combined at grain maturity (>45 DAH). Prior to analysis, spikes were dried at 36°C for 7d and threshed. Seeds were ground using a Retsch mill ZM-200 fitted with a <0.5 mm screen (Retsch GmbH, Newtown, PA) after equilibrating to ambient relative humidity for 7d. Whole meals were stored at ambient conditions for up to 7d until extraction. Moisture content was estimated from a random set of subsamples for each sampling date (AACC method 44-19). Briefly, weighed grain samples (≤ 3 g) were oven-dried at 130°C for 65 minutes, then cooled in a desiccator and weighed again to three significant figures. For the lutein loss test, 18 frozen samples of spikes were randomly selected from 28 and 35 DAH sample sets of Kernen (2007) and divided. One set was dried as described before, and the other was freeze-dried immediately prior to carotenoid analysis.

3.2 Total Yellow Pigment Analyses

Total yellow pigment was assessed on mature grain from individual plots using AACC-approved method 14-50 (AACC, 2000). Briefly, grain was ground in a UDY Cyclone Sample Mill (UDY Corporation, Fort Collins, Colorado) fitted with a 1 mm screen and water-saturated butyl alcohol (40 mL) was added to 8 g of whole meal (14% moisture basis), shaken and extracted for 16 hours. Extracts were then filtered through Whatman No. 1 filter paper, and absorbance measured at 435 nm using a Microplate Reader (BioRad, CA, USA). Two individual absorbance measurements per extracted sample were recorded and values were averaged and converted to yellow pigment concentration ($\mu g\ g^{-1}$) using the extinction coefficient for β-carotene (AACC 2000). Total YP is reported on a 14% moisture basis.

3.3 Carotenoid Extraction, Identification and Quantification

To determine a suitable carotenoid extraction protocol, two preliminary lutein extraction tests were performed in triplicate.For the first test, four extraction solvents (80% aqueous ethanol; water-saturated *n*-butanol; 1:1 methanol: dichloromethane and 5:5:5:3 methanol: ethanol: dichloromethane: water; all solvents with 0.1% BHT w/v as antioxidant) were evaluated for their lutein recovery. All extractions were carried out by shaking at 300 rpm for 1 hour at 30°C. For the second test, four extraction methods (AACC 14-50 as a control; shaking at 300 rpm for 1 hour at 30°C followed by centrifugation, performed either once or twice; sonication for 1 hour followed by centrifugation) were evaluated with water saturated *n*-butanol (WSB). The two experiments were analyzed independently. In the case of the extraction performed twice, volumes of both supernatants after centrifugation were measured and pooled. All methods were evaluated on whole meals of three durum lines (low-YP 'Demetra', medium-YP 'W9262-260*D3' and high-YP 'Novadur') from the field study. Approximately 1 g of whole meal was extracted with 5 mL of solvent, submitted to the respective extraction method and filtered. The lutein yield, in $\mu g\ g^{-1}$, was measured by HPLC using the isocratic mobile phase system described by Hu et al. (2007), with the lutein yield from the AACC 14-50 method set as a control. Based on these results, a standardized protocol was developed as follows.

For each sample from immature spikes or mature grain, 0.5-1.5 g of whole meal was mixed with 3-5 mL CH_3OH:CH_2Cl_2 (1:1 v/v, 0.1% BHT w/v) in polypropylene or borosilicate tubes (VWR Canada) under dim light (unlit fume hood in a room with artificial fluorescent light) and shaken at 300 rpm (30°C) on a gyratory shaker for 1 hour. Extracts were then centrifuged for 4 minutes at 13500 *g,* filtered through 0.2 µm PVDF syringe filters and stored in glass inserts inside amber glass vials at -80°C for up to two weeks until HPLC analysis. Tomato paste and fresh passion fruit pulp were extracted using the same procedure as a qualitative source of lycopene and ζ-carotene, respectively. Carotenoid quantification for Kernen (2007) was later aborted for all immature samples (except the degradation test). This was because meal samples were inadvertently stored for longer than the maximum 7d period, and progressive lutein degradation was observed. Therefore, mature grain data for this environment was analyzed separately and presented in parallel with the analyses of other environments.

Carotenoids were separated on a YMC C30 column (250 x 4.6 mm i.d, 3 µm; YMC America, Newtown, PA), preceded by a YMC C30 guard column (20 x 4 mm i.d, 3 µm), using a Waters Alliance 2695 separations module (Waters Corporation, Milford, MA). The analytical column was replaced twice during the course of the study because of irreversible contamination with unknown compounds that were insoluble in the mobile phase or in more non-polar solvents (100% CH_2Cl_2). Columns were replaced because of increasing back-pressure and poor chromatography. A guard column (YMC Carotenoid C30, 20 x 4 mm, 3 µm), replaced as necessary, was included with the second and third analytical columns. Data was collected at 1 nm increments with a Waters 2998 photodiode array detector set in the range of 190-600 nm. For all mature grain samples, extracts were eluted with an isocratic separation using solvent A (58:30:10.5:1.5 CH_3OH:CH_2Cl_2: CH_3CN: H_2O; "mobile phase 1") (Hu et al., 2007). This was later modified (Shi and Chen, 1999) for immature samples from Kernen 2008 to enable separation of lutein from chlorophyll *b*. This protocol ("mobile phase 2") utilized solvents A and B (100% CH_3OH) with a 35-min gradient. The gradient was: 50% A, 0-10 min, 1.1 mL/min; 100% A, 25 min, 1.3 mL/min; hold 5 min; 50% A, 35 min, 1.3 mL/min. However this method did not adequately separate chlorophyll *a* from zeaxanthin, particularly at high concentrations of the former. Therefore, a third, more

effective protocol ("mobile phase 3") was developed, based on the procedure used by Lakshminarayana et al. (2005), for immature grain extracts from the remaining environments. This mobile phase system comprised 58:22:20 CH_3CN: CH_3OH: CH_2Cl_2 at a flow rate of 0.65 mL/min, 0-14 min; 0.3 mL/min, 14.1 min; hold 3 min; 1 mL/min at 18 min; hold, 18-55 min. This separated lutein, zeaxanthin and both chlorophylls. This method had initially been rejected in view of the rising cost of acetonitrile (CH_3CN) during the 2008 global recession. An alternative protocol using diverse gradients of ethyl acetate in methanol was also rejected because of poor separation (data not shown).

On the first two analytical columns, a regeneration protocol (3 to 4 100 µL injections of dimethyl sulfoxide every 10 min in 100% methanol at 0.2 mL/min) was used with diminishing success in column cleanup. On the third analytical column, a more effective and repeatable regeneration protocol was designed and incorporated after every 15-20 samples to enable complete elution of unknown column contaminants. The guard column was reversed and attached to the outlet before equilibrating to 100% methanol at 38°C. The columns were then rinsed (0.25 mL/min for 120 min) with a 1:1 (v/v) solution of 2-propanol and 1% aqueous acetic acid (w/v), followed by a gradient rinse (0.2 to 0.7 mL/min over 120 min) with 100% deionized water at 38°C. The columns were then equilibrated with 100% acetonitrile at 27°C before reverting to mobile phase 3.

All mobile phase solvents were of HPLC grade (EMD Chemicals, Gibbstown, NJ). For all samples, a single injection was made with volumes ranging from 10 to 80 µL in a 300 µL sample loop. The sample chamber was maintained at 10°C and samples were injected within 24 hours. Stock and working solutions were prepared in extraction solvent, except for carotene stock solutions (1:3 v/v CH_3OH:CH_2Cl_2, 0.1% BHT).

Standards of *E*-lutein (89% purity), *E*-zeaxanthin (98% purity), *E*-α-carotene (96% purity) (ChromaDex, Irvine, CA, USA), *E*-β-carotene (95% purity) and chlorophylls *a* and *b* (95% purity) (Sigma-Aldrich Canada, Oakville, ON) were used to construct linear standard curves by injecting 4 to 80 ng of lutein (4, 10, 20, 40, 80 ng intervals) (lutein) or 2 to 40 ng (others) . Chromatographic peaks were identified by comparing retention times and absorbance spectra to those of standards. All other peaks

were tentatively identified when possible, based on spectral characteristics. A peak was identified as a putative carotenoid if characteristic triple maxima were observed in the absorbance spectrum (Britton, 1995) while retention time was used to determine if the peak represented a xanthophyll (≤ 20 min) or a carotene (>25min). Polar carotenes eluting relatively quickly, such as ζ-carotene, were easily identified by their distinct spectra (Britton, 1995). These carotenoid-like peaks were quantified with the *E*-lutein standard curve. Using peak areas, all carotenoids were quantified at 447 nm, the absorbance maximum for *E*-lutein. For lutein, α-carotene and β-carotene, peak identification was confirmed by spiking durum samples with the respective standards. Chromatographic peaks were integrated to the baseline when possible. If baseline separation was not possible, peaks were integrated to the midpoint of adjacent minima with neighbouring peaks. Peak purity was confirmed by verifying the consistency of absorbance spectra throughout the integrated area. Impure peaks were not quantifiedUnless otherwise denoted as '*Z*', all carotenoids that are indicated here imply the all-*E* form. For the sake of clarity, 'concentration' denotes μg g^{-1} and 'content' indicates ng $kernel^{-1}$. The latter was estimated by multiplying the mean kernel weight (mg, dry basis) by the concentration (μg g^{-1}, dry basis). Kernel weight was determined for each sample by weighing 100 kernels. Carotenoid and YP concentration estimates are restricted to mature grain samples while carotenoid content estimates are reported for all sampling dates. All carotenoid estimates are reported on a dry-matter basis. However, all carotenoid percentages of YP presented in this paper are based on YP reported on a 14% moisture basis, for consistency with previous literature on YP. These can be adjusted to real percentages by multiplying by a factor of 0.86.

3.4 Statistical Analysis

Combined analysis of variance (across three environments: Kernen 2008, SC 2007 SC 2008) was performed using the PROC MIXED procedure of SAS (Version 9.1) (SAS Institute, Cary, NC). Genotypes and sampling dates were considered fixed factors. Data from each environment were also analyzed separately. Unless indicated otherwise, all estimates and proportions were derived from the combined analysis of three environments (Ker 2008, SC 2007, and SC 2008). YP and carotenoid data from mature samples of Kernen 2007 are also reported but were analyzed alone because no grain

development data was obtained from this environment. Significant differences were analyzed by Fisher's LSD test at $\alpha=0.05$. Pearson's correlations were performed in Minitab (12.1).

4. RESULTS

4.1 Lutein Extraction and Loss Tests

The ANOVAs for the lutein extraction study are presented in Tables 2 and 3, while the least squares means are shown in Table 4. The screened extraction solvents were not significantly different at the 5% level (p=0.07), and a double solvent extraction by shaking yielded significantly more lutein than all other methods (Table 4). Compared to a single extraction, the lutein yield was greater by 6%. Because there were no interactions with genotype, lutein yields are presented as a mean of all genotypes. A good correlation (r = 0.96) was observed between lutein concentration in conventionally-dried and freeze-dried samples. The mean lutein concentration in conventionally-dried samples was 14% lower than in freeze-dried samples.

Table 2. F-tests for fixed effects from analysis of variance (ANOVA) of lutein concentrations in three genotypes (grown at Kernen, 2007) from four solvents.

Source	**Lutein yield (F value)**
Genotype	405.3***
Solvent	2.63
Genotype x solvent	0.56

*p<0.05; **p<0.01; ***p<0.001

Table 3. F-tests for fixed effects from analysis of variance (ANOVA) of lutein concentrations in three genotypes (grown at Kernen, 2007) from four extraction methods.

Source	**Lutein yield (F value)**
Genotype	2632.3***
Extraction	12.06***
Genotype x extraction	0.93

*p<0.05; **p<0.01; ***p<0.001

Table 4. Least Squares Means from lutein extraction test

Solvent	**Lutein Yield (µg g^{-1})**
80% EtOH	3.036
MeOH:DCM	3.267
MeOH:DCM:EtOH:H_2O	3.138
WSB	2.892
LSD (p=0.05)	**ns**

Extraction Method (WSB)	**Lutein Yield (µg g^{-1})**
Shaking (double extraction)	3.085[a]
Shaking (single extraction)	2.892[b]
Sonication	2.751[b]
AACC 14-50	2.903[b]
LSD (p=0.05)	**0.174**

4.2 Carotenoid Identification in Mature and Immature Grain Samples

Typical sample chromatograms are shown in Figure 7 for mature and immature grain and the characteristics of major peaks are summarized in Table 5. In mature grain, all-*E* forms of lutein, zeaxanthin, α-carotene and β-carotene were easily identified and were quantified using standard curves generated from primary standards of each carotenoid. Several unidentifiable peaks were also detected but, in the case of mature grain samples, were quantified because they exhibited typical carotenoid-like spectra (Britton, 1995; Liaaen-Jensen and Lutnæs, 2008). These peaks could not be resolved accurately in immature grain samples because of co-elution with chlorophylls. Six unknown xanthophyll-like peaks were identified in mature grain samples. Four of these peaks exhibited *Z* absorbance maxima (Table 5). The two more distinctly putative *Z*-isomers (peaks 3 and 4) exhibited *Z*-peak shifts of 143 nm and 144 nm and hypsochromic shifts, relative to *E*-lutein, of 5 and 7 nm, respectively. The other two (peaks 7 and 8) exhibited very low *Z* maxima, as seen in the A_b/A_{II} (Q) ratios, and respective hypsochromic shifts of 5 and 4 nm. Their respective Z-peak shifts were 132 and 141 nm relative to *E*-lutein, but 138 and 149 nm relative to *E*-zeaxanthin. The III/II ratios, indicating fine structure, ranged from over 100% for peaks 1 and 2, to <50% for peaks 3 and 4.

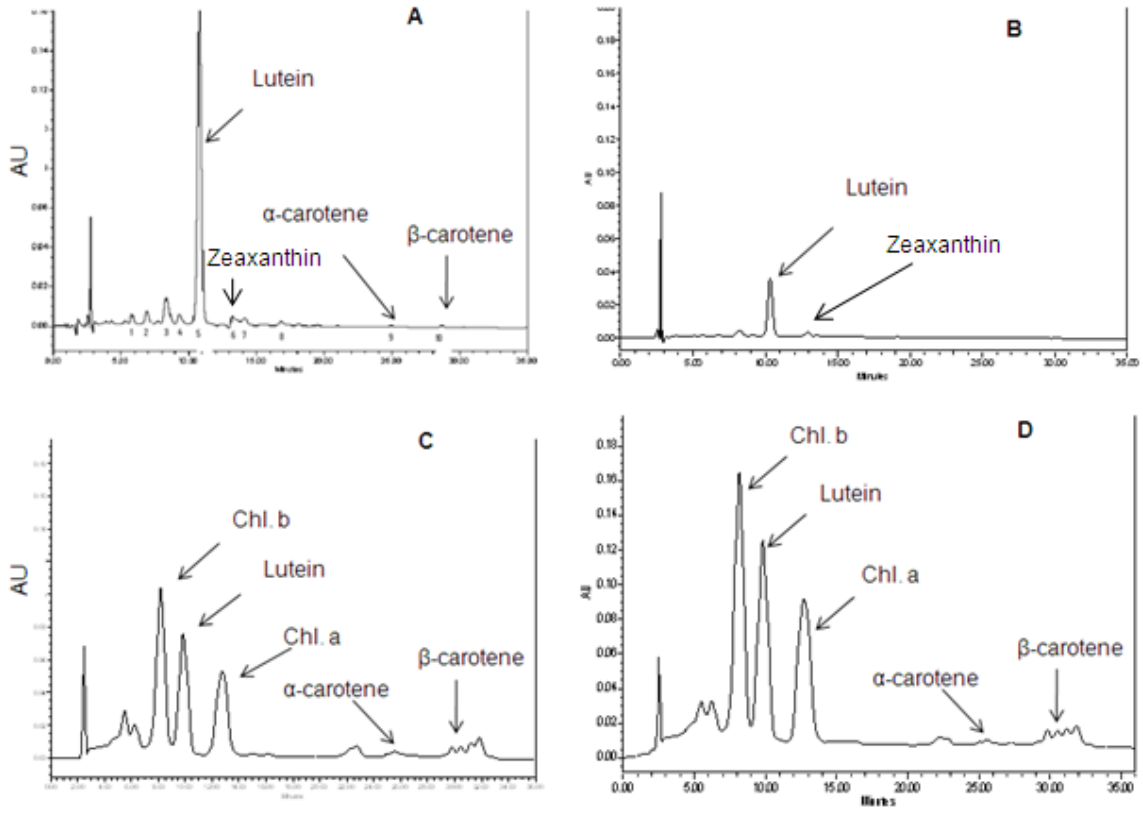

Figure 7. Typical sample chromatograms of high-YP line '940435' at grain maturity (A) and at 14 days after heading (DAH) (C); and of low-YP line 'A9831&DC*1' at grain maturity (B) and at 14 DAH (D). All-(*E*) forms of lutein, zeaxanthin, α-carotene and β-carotene, and chlorophyll *a* and chlorophyll *b* peaks are identified. HPLC conditions (mobile phase 2) are described in the text.

Table 5. List of chromatographic peaks, their observed spectra and spectral parameters (where applicable) from a typical HPLC chromatogram of a mature grain sample from durum breeding line '940435' grown at Kernen in 2008. Carotenoid peaks (1 to 10) are shown in order of increasing retention time. HPLC conditions are described in the text.

Peak Number	Peak Identification	λmax (nm)			A_b/A_{II} ratio (%)	Z band λ (nm)	III/II Ratio (%)
		I	II	III			
1	Putative xanthophyll	418	441	471	-	-	102
2	Putative xanthophyll	401	424	449	-	-	115
3	Putative xanthophyll	420	442	467	42	332	32
4	Putative xanthophyll	419	440	466	43	331	50
5	*E*-Lutein	428	447	475	-	-	66
6	*E*-Zeaxanthin	432	453	481	-	-	100
7	Putative xanthophyll	419	442	470	8	343	71
8	Putative xanthophyll	417	443	471	9	334	60
9	*E*-α-carotene	422	449	478	-	-	59
10	*E*-β-carotene	430	456	483	-	-	32
11	Chlorophyll *b*	-	470	-	-	-	-
12	Chlorophyll *a*	-	433	-	-	-	-

In most immature grain samples (14 through 35 DAH), none of the carotenoids (except lutein) could always be quantified with precision as these co-eluted with either chlorophyll *a* or *b* or other unknown compounds. As well, several unknown compounds were identified, which were not the same as the unidentified putative carotenoids observed in mature grain samples (Figure 7; Table 5). A common spectral characteristic of these unknown peaks was the presence of a single, asymmetric absorbance maximum ranging between 400 and 450 nm. Their polarities were highly variable, with some peaks eluting before chlorophyll *b* and others eluting after β-carotene. Because of the absence of distinct carotenoid-like spectra, these peaks were suspected to not be carotenoids and were not quantified. Lycopene and ζ-carotene were not detected in any

of the samples analyzed. Therefore, only lutein was quantified in all of these samples. Zeaxanthin, α-carotene and β-carotene were quantified for all immature grain samples except those at 14 DAH.

4.3 Total Yellow Pigment

Total YP concentration among the 13 genotypes was variable in all three environments, but the genotype x environment interaction was not significantly different from the residual variance ($p>0.05$; Table 6). Therefore, a combined analysis was used. This was confirmed with correlation analysis. Pearson's correlation coefficients of all genotype LSmeans among environments were high (0.97-0.98; $p<0.001$), as were Spearman's rank correlation coefficients (Table 7). YP and carotenoid data from mature grain in these three environments is presented in Table 8. The ANOVA from Kernen (2007) is presented separately in Table 9. Genotype was the only significant effect in this environment. The average monthly temperature and precipitation during the growing season (May-September) is provided for each environment in Appendix 1.

Averaged over all three environments, Demetra had the lowest YP concentration (5.19 μg g^{-1}) and Novadur the highest (14.32 μg g^{-1}) (Table 8). Based on consistent expression of YP concentration, the 13 genotypes could be classified into one of three groups (Table 8). These classifications were made based on the absence of significant differences amongst genotypes in each group. The average YP of the high group expressed 3.51 μg g^{-1} more YP than the intermediate group. The low group had 4.69 μg g^{-1} less YP than the average YP of genotypes in the intermediate group. The range in YP was greatest in the intermediate YP group, but no significant differences were detected among cultivars classified into this group at any of the three environments. Data from Kernen (2007) is presented in Table 10. Similar to the combined analysis, high, intermediate and low-YP groups were significantly different from one another in YP content, and no significant differences were observed within a group.

Table 6. Variance estimates for random effects and F tests for fixed effects from combined analysis of variance (ANOVA) of yellow pigment (YP) concentration (14% moisture basis), kernel weight (KWT), and carotenoid concentrations (lutein, zeaxanthin, α-carotene, β-carotene and summed unknown carotenoids) in mature grain samples of 13 genotypes from three environments (Kernen 2008, SC 2007, SC 2008) using PROC MIXED.

Random Effects	**YP**	**KWT**	**Lutein**	**Zeaxanthin**	**α-carotene**	**β-carotene**	**Unknowns**
Environment	0.424	109.5	0	0.019	0	0	0
Environment (Rep)	0.036	0.876	0.063	0.001	0	0	0
Genotype*Environment	0.431*	1.710	0.031	0.001	0	0.0008**	0.005
Residual	0.577***	0.969***	0.039***	0.001***	0.0005***	0.0005***	0.034***
Fixed Effects							
Genotype	59.65***	4.18**	143.3***	0.90	41.2***	3.07**	17.95***

*p<0.05; **p<0.01; ***p<0.001

Table 7. Pearson's and Spearman's rank correlation coefficients between YP concentrations of 13 genotypes in three environments (Kernen 2008, SC 2007, and SC 2008)

R value (Pearson's)	**K 08**	**SC 07**	**SC 08**
K 08	-	0.981***	0.975***
SC 07		-	0.972***
R value (Spearman's)			
K 08	-	0.956***	0.940***
SC 07		-	0.951***

*p<0.05; **p<0.01; ***p<0.001

Table 8. LS means of total yellow pigment concentration (YP; 14% moisture basis) and concentrations of carotenoids at maturity of 13 cultivars and breeding lines (dry matter basis) grown at three environments (SC 2007 and 2008, Kernen 2008) in a replicated randomized complete block design. Values in parenthesis represent the % of total yellow pigment for each carotenoid. Correlation is of individual carotenoids with YP.

Genotype	YP (µg g^{-1})	*E*-Lutein (µg g^{-1})		*E*-Zeaxanthin (µg g^{-1})		*E*-α-carotene (µg g^{-1})		*E*-β-carotene (µg g^{-1})	
High Pigment Group									
2805	13.75	7.42	(54.0)	0.35	(2.5)	0.14	(1.0)	0.11	(0.80)
940435	13.60	7.43	(54.6)	0.38	(2.8)	0.17	(1.3)	0.09	(0.66)
Novadur	14.32	7.56	(52.8)	0.31	(2.2)	0.16	(1.1)	0.13	(0.91)
Mean	**13.89**	**7.47**	**(53.8)**	**0.35**	**(2.5)**	**0.16**	**(1.1)**	**0.11**	**(0.79)**
Intermediate Pigment Group									
Commander	10.96	5.99	(54.7)	0.33	(3.0)	0.09	(0.8)	0.11	(1.00)
DT696	9.94	4.96	(49.8)	0.34	(3.5)	0.02	(0.2)	0.05	(0.50)
DT707	9.76	4.87	(49.9)	0.34	(3.5)	0.05	(0.5)	0.06	(0.61)
Kofa	11.01	6.08	(55.2)	0.39	(3.5)	0.03	(0.3)	0.07	(0.63)
Strongfield	10.73	5.67	(52.8)	0.34	(3.2)	0.03	(0.3)	0.07	(0.65)
W9262-260D3	9.68	5.21	(53.8)	0.33	(3.4)	0.04	(0.4)	0.06	(0.62)
Mean	**10.38**	**5.46**	**(52.6)**	**0.34**	**(3.3)**	**0.03**	**(0.3)**	**0.07**	**(0.67)**
Low Pigment Group									
A0022&D509	6.16	2.26	(36.6)	0.35	(5.7)	0.00	(0.1)	0.05	(0.81)
A0600B-020	5.88	2.30	(39.2)	0.39	(6.6)	0.00	(0.0)	0.04	(0.68)
A9831-DC*1	5.51	2.16	(39.2)	0.32	(5.7)	0.01	(0.2)	0.03	(0.54)
Demetra	5.19	1.99	(38.4)	0.27	(5.3)	0.00	(0.0)	0.03	(0.58)
Mean	**5.69**	**2.18**	**(38.3)**	**0.33**	**(5.9)**	**0.01**	**(0.1)**	**0.04**	**(0.70)**
LSD (α=0.05)	1.38	0.49		ns		0.03		0.05	
Correlation (r)	-	0.99***		ns		0.88***		0.89***	

4.4 Carotenoid Quantification in Mature Grain Samples

At maturity, the predominant carotenoid in all samples was lutein, with significant differences ($p<0.05$) detected among the genotypes. Similar to total YP, the environment x genotype interaction was not statistically significant ($p>0.05$). The correlation between total yellow pigment and lutein was 0.99 ($p<0.01$). Expressed as a proportion of total YP, lutein accounted for approx. 54% of the total YP in the high and intermediate pigment groups (Table 8). In contrast, only 38.3% of total YP was lutein in the low pigment group (Table 8). Similar patterns were seen in Kernen (2007) (Table 10). In mature grain samples, zeaxanthin was the second-most abundant confirmed carotenoid, but no significant differences were detected among genotypes (Table 8). Correlation between zeaxanthin and total YP was not significant ($P>0.05$; Table 8 and Table 10). The low YP accumulators had a greater proportion of yellow pigment in the form of zeaxanthin (5.9%) compared to the intermediate (3.3%) and high (2.5%) pigment groupings (Table 8). In Kernen 2007, approximately similar patterns existed (Table 10). Traces of α-carotene and β-carotene were detected in all genotypes, but together accounted for less than 2% of the total yellow pigment. However, the concentrations of both α-carotene and β-carotene were highly correlated with total YP concentration in all environments (Table 8, Table 10).

Table 9. Variance estimates for random effects and F tests for fixed effects from analysis of variance (ANOVA) of yellow pigment (YP) concentration (14% moisture basis), kernel weight (KWT), and carotenoid concentrations (lutein, zeaxanthin, α-carotene, and β-carotene) in mature grain samples of 13 genotypes from one environment (Kernen 2007) using PROC MIXED.

Source	YP	KWT	Lutein	Zea-xanthin	α-carotene	β-carotene
Random Effects						
Rep	0.092	0	0.021	0	0	0
Residual	1.306**	1.231**	0.034**	0.0004**	0.0001**	0.0003**
Fixed Effects						
Genotype	26.85***	13.58***	301.08***	3.35*	32.2***	8.55***

*p<0.05; **p<0.01; ***p<0.001

Table 10. LSmeans of total yellow pigment concentration (YP; 14% moisture basis) and concentrations of carotenoids at maturity of 13 cultivars and breeding lines (dry matter basis) grown at one environment (Kernen 2007) in a replicated randomized complete block design. Values in parenthesis represent the % of total yellow pigment for each carotenoid. Correlation is of individual carotenoids with YP.

Line	YP (µg g^{-1})	*E*-Lutein (µg g^{-1})		*E*-Zeaxanthin (µg g^{-1})		*E*-α-carotene (µg g^{-1})		*E*-β-carotene (µg g^{-1})		Zea/Lutein (x100)	α-carotene/ lutein (x100)	β-carotene/ Zea (x100)
High Pigment Group												
2805	15.01	8.71	(58.0)	0.32	(2.1)	0.08	(0.53)	0.12	(0.80)	3.67	0.92	37.50
940435	13.92	7.61	(54.7)	0.28	(2.0)	0.19	(1.36)	0.09	(0.65)	3.68	2.50	32.14
Novadur	14.40	7.62	(55.3)	0.27	(1.9)	0.11	(0.76)	0.11	(0.76)	3.54	1.44	40.74
Mean	**14.44**	**7.98**	**(56.0)**	**0.29**	**(2.0)**	**0.13**	**(0.90)**	**0.11**	**(0.76)**	**3.63**	**1.62**	**19.21**
Intermediate Pigment Group												
Commander	11.52	6.01	(52.2)	0.27	(2.3)	0.04	(0.35)	0.09	(0.78)	4.49	0.67	33.33
DT696	11.07	5.12	(46.3)	0.28	(2.5)	0.04	(0.36)	0.06	(0.54)	5.47	0.78	21.43
DT707	10.24	5.07	(49.5)	0.29	(2.8)	0.03	(0.29)	0.07	(0.68)	5.72	0.59	24.14
Kofa	10.65	5.99	(56.2)	0.32	(3.0)	0.03	(0.28)	0.07	(0.66)	5.34	0.50	21.88
Strongfield	10.54	5.12	(48.6)	0.31	(2.9)	0.02	(0.19)	0.07	(0.66)	6.05	0.39	22.58
W9262-260D3	10.20	4.99	(48.9)	0.28	(2.7)	0.03	(0.29)	0.05	(0.49)	5.61	0.60	17.86
Mean	**10.70**	**5.38**	**(50.3)**	**0.29**	**(2.7)**	**0.03**	**(0.28)**	**0.07**	**(0.65)**	**5.45**	**0.59**	**12.06**
Low Pigment Group												
A0022&D509	5.44	2.15	(39.5)	0.32	(5.9)	0.00	(0.0)	0.05	(0.92)	14.88	0.00	15.63
A0600B-020	5.81	2.45	(42.2)	0.31	(5.3)	0.00	(0.0)	0.03	(0.52)	12.65	0.00	9.68
A9831-DC*1	5.33	2.35	(44.1)	0.27	(5.1)	0.00	(0.0)	0.02	(0.38)	11.49	0.00	7.41
Demetra	4.91	1.91	(38.9)	0.23	(4.7)	0.00	(0.0)	0.02	(0.41)	12.04	0.00	8.70
Mean	**5.37**	**2.22**	**(41.3)**	**0.28**	**(5.2)**	**0.00**	**(0.0)**	**0.03**	**(0.56)**	**12.77**	**0.00**	**5.18**
LSD (α=0.05)	1.49	0.37		0.04		0.03		0.03		1.56	0.33	10.2
Correlation (r)	-	0.99*		ns		0.78**		0.93***				

On average, the genotypes classified into the high YP group had more α-carotene than the intermediate and low YP groups. A correlation matrix for these four carotenoids is shown in Table 11. All correlations were significant except those between zeaxanthin and any other carotenoid. Correlations from Kernen (2007) are presented in Table 12, where a similar pattern was observed.

Table 11. Correlation matrix between LSmeans of lutein, zeaxanthin, α-carotene, and β-carotene in three environments (Kernen 2008, SC 2007 and SC 2008)

	E-Lutein	*E*-Zeaxanthin	*E*-α-carotene	*E*-β-carotene
E-Lutein	.	0.232	0.854***	0.878***
E-Zeaxanthin		.	0.091	0.080
E-α-carotene			.	0.881***

Table 12. Correlation matrix between LSmeans of lutein, zeaxanthin, α-carotene, β-carotene in Kernen 2007.

	E-Lutein	*E*-Zeaxanthin	*E*-α-carotene	*E*-β-carotene
E-Lutein	.	0.179	0.780**	0.936***
E-Zeaxanthin		.	-0.076	0.270
E-α-carotene			.	0.669*

The six unknown carotenoid-like peaks identified in mature grain samples of all genotypes in Kernen (2008) and SC (2008), likely xanthophylls because of their spectra and retention times (Figure 7 and Table 5), were summed and are presented as "unknowns" (Table 13). Data from the 2007 environments was not included because not all peaks could be quantified accurately. Similar to lutein, the total concentration of unknown compounds was correlated with total YP (0.96; $P<0.01$) and was highest in the genotypes expressing the most yellow pigment. Based on the standard curve for *E*-lutein, the unidentified carotenoids represented 11.6 % of the total yellow pigment in the high grouping, similar to the 12.5% observed in the intermediate pigment group. Only 10.0% of the total YP was unknown carotenoids in low YP genotypes (Table 13). A correlation matrix comparing these six unknowns to YP and the four identified

carotenoids is presented in Table 14. Each of these unknowns was significantly positively correlated to YP, as well as to lutein. Zeaxanthin's only significant correlation was with peak 2. Except for peak 1, all were significantly positively correlated with both carotenes. Similarly, significant positive correlations existed amongst the unknown carotenoids, except those involving peak 1.

Table 13. LSmeans of total yellow pigment concentration (YP; 14% moisture basis) and concentrations of total unknown carotenoids (expressed as *E*-lutein) at maturity of 13 cultivars and breeding lines (dry matter basis) grown at two environments (Kernen 2008 and SC 2008) in a replicated randomized complete block design. Values in parenthesis represent the % of total yellow pigment for unknown carotenoids.

Genotype	**YP ($\mu g\ g^{-1}$)**	**Unknowns ($\mu g\ g^{-1}$)**	
High Pigment Group			
2805	13.37	1.50	(11.2)
940435	13.31	1.72	(12.9)
Novadur	13.3	1.43	(10.8)
Mean	**13.4**	**1.55**	**(11.6)**
Intermediate Pigment Group			
Commander	10.78	1.36	(12.6)
DT696	9.78	1.25	(12.8)
DT707	9.54	1.17	(12.3)
Kofa	10.45	1.27	(12.2)
Strongfield	10.28	1.33	(12.9)
W9262-260D3	9.27	1.11	(12.0)
Mean	**10.02**	**1.25**	**(12.5)**
Low Pigment Group			
A0022&D509	5.92	0.64	(10.8)
A0600B-020	5.95	0.65	(10.9)
A9831-DC*1	5.44	0.52	(9.6)
Demetra	5.32	0.47	(8.8)
Mean	**5.66**	**0.57**	(**10.0**)
LSD (α=0.05)	1.37	0.27	
Correlation (r)	-	0.96***	

Table 14. Correlation matrix between LSmeans of YP, lutein, zeaxanthin, α-carotene, β-carotene and six putative carotenoids from two environments (Kernen 2008, SC 2008). Peaks are numbered in order of increasing retention time. Peaks 1-4 and 7-8 are unidentified or tentatively identified in the text. These unknown carotenoids were not quantified in the 2007 environments.

		Carotenoid Peaks									
		1 Unknown	**2 Unknown**	**3 Unknown**	**4 Unknown**	**5 Lutein**	**6 Zeaxanthin**	**7 Unknown**	**8 Unknown**	**9 α-carotene**	**10 β-carotene**
Carotenoid Peaks	**Total YP**	0.503	0.965***	0.961***	0.951***	0.994***	-0.375	0.938***	0.919***	0.874***	0.927***
	1	.	0.637*	0.549	0.532	0.555*	-0.087	0.612*	0.571*	0.368	0.406
	2		.	0.923***	0.898***	0.973***	0.803**	0.906***	0.862***	0.803**	0.867***
	3			.	0.975***	0.971***	-0.274	0.955***	0.926***	0.760**	0.875***
	4				.	0.955***	-0.377	0.986***	0.969***	0.775**	0.849***
	5 (Lutein)					.	-0.368	0.951***	0.926***	0.838**	0.838**
	6 (Zeaxanthin)						.	-0.354	-0.358	-0.465	-0.328
	7							.	0.986***	0.772**	0.844**
	8								.	0.802**	0.850***
	9 (α-carotene)									.	0.896***

*p<0.05; **p<0.01; ***p<0.001

At maturity, statistically significant ($P<0.05$) variation in seed weight was evident among the 13 genotypes (Table 15). Averaged over all three environments, the range in seed weight was 8 mg seed^{-1} (Table 15). The correlations between seed weight and total yellow pigment was not statistically significant ($p>0.05$). To remove the confounding effects of seed size on concentration estimates of pigment or carotenoids, the average kernel content (ng kernel^{-1}) of each identified carotenoid was estimated (Table 15). The content of lutein, α-carotene and β-carotene were significantly different among the three groups, with the lowest content observed in genotypes expressing low YP (Table 15). No significant differences in zeaxanthin content were detected among the 13 genotypes evaluated in this study. However, the zeaxanthin to lutein ratio in mature grain samples was highest in the low YP genotypes, with a significant negative correlation ($r = -0.92$) with yellow pigment (Table 15). Similar patterns were seen in Kernen 2007, where a negative correlation ($r= -0.94$) existed between the zeaxanthin/lutein ratio and YP (Table 10). In contrast, the α-carotene/lutein ratio was greatest in high pigment accumulators, and was significantly different among all three pigment groupings (Table 15). Although the β-carotene: zeaxanthin ratio was also greatest in high YP genotypes, some intermediate pigment genotypes (DT696 and Commander), had ratios similar to 940435 (Table 15). With the exception of Commander, intermediate-YP genotypes were not significantly different from low-YP genotypes. At Kernen 2007, the α-carotene: lutein and β-carotene: zeaxanthin ratios also showed a similar pattern to the three-environment analysis, although the differences between YP groups were smaller for the β-carotene: zeaxanthin ratio.

Table 15. Kernel weight (mg) and content of identified carotenoids (ng seed^{-1}) at maturity for 13 genotypes with high, intermediate, and low expression of yellow pigment concentration in three environments (Ker 2008, SC 2007, SC 2008).

Genotype	KWT (mg)	Lutein (ng sd$^{-1)}$	Zeaxanthin (ng sd^{-1})	α-carotene (ng sd$^{-1)}$	β-carotene (ng sd$^{-1)}$	Zea / Lutein (x100)	α-carotene / Lutein (x100)	β-carotene / Lutein (x100)	β-carotene / Zea (x100)
High Pigment Group									
2805	34.5	256	12.1	4.72	4.11	4.32	1.84	1.47	42.4
940435	37.6	279	14.1	6.51	3.37	5.07	2.35	1.20	37.2
Novadur	34.7	262	10.8	5.44	4.49	4.10	2.09	1.75	47.0
Mean	**35.6**	**266**	**12.3**	**5.55**	**4.01**	**4.63**	**2.09**	**1.47**	**42.2**
Intermediate Pigment Group									
Commander	39.1	234	12.8	3.52	4.97	5.45	1.50	1.86	43.3
DT696	38.5	191	13.2	0.90	1.97	6.93	0.50	1.12	23.0
DT707	33.5	163	11.3	1.56	1.89	6.92	0.97	1.16	20.8
Kofa	37.6	229	14.5	1.25	2.87	6.36	0.55	1.14	20.6
Strongfield	36.5	207	12.5	1.10	2.45	6.06	0.53	1.21	22.1
W9262-260D3	33.4	174	11.0	1.34	1.87	6.34	0.76	1.08	19.9
Mean	**36.4**	**200**	**12.6**	**1.61**	**2.67**	**6.31**	**0.61**	**1.26**	**24.4**
Low Pigment Group									
A0022&D509	31.1	70	11.0	0.10	1.21	15.66	0.13	2.26	13.2
A0600B-020	36.2	83	14.1	0.00	1.39	16.93	0.00	2.02	11.3
A9831-DC*1	36.2	78	11.5	0.36	0.97	14.66	0.47	1.56	9.0
Demetra	37.6	75	10.3	0.00	0.75	13.71	0.00	1.52	10.5
Mean	**35.3**	**77**	**11.7**	**0.12**	**1.08**	**15.30**	**0.16**	**1.84**	**10.8**
LSD (α=0.05)	3.2	39	ns	0.81	1.48	4.05	0.46	ns	15.0

4.5 Accumulation of Carotenoids during Grain Fill

Repeated measures analysis of lutein content (ng kernel^{-1}) during the grain filling period was conducted on the yellow pigment groups (classes). The ANOVA revealed a significant class x environment x sampling time interaction ($P<0.05$; Table 16), so data were analyzed and presented for individual environments (Table 17; Figure 8).

Table 16. Variance estimates for random effects and F tests for fixed effects from combined analysis of variance (ANOVA) of kernel weight (KWT) and carotenoid content (lutein, zeaxanthin, α-carotene and β-carotene) during grain fill in samples of three genotype classes (low-YP, intermediate-YP, high-YP) from three environments (Kernen 2008, SC 2007, SC 2008), using PROC MIXED.

Random Effects	**KWT**	**Lutein**	**Zeaxanthin**	**α-carotene**	**β-carotene**
Environment	0	0	0	13.01	14.27
Environment (Rep)	0.316	0	0	0	0.076
Class*Environment	0.863*	0	0	0.197	0.238
Environment*Sampling Date	56.43*	1780*	6.485	4.158	5.048
Class*Sampling Date* Environment	2.09***	816.9**	6.479	0.459	0.617
Residual	3.12***	857.9***	2.389***	4.917***	5.718***
Fixed Effects					
Class	3.19**	49.35***	9.67*	7.28	3.88
Sampling Date	7.68**	3.15	1.31	0.13	0.07
Class*Sampling Date	2.06**	5.42**	0.71	4.96*	1.40

*p<0.05; **p<0.01; ***p<0.001

Table 17. Variance estimates for random effects and F tests for fixed effects from individual analysis of variance (ANOVA) of kernel weight (KWT) and carotenoid content (lutein, zeaxanthin, α-carotene and β-carotene) during grain fill in samples of three genotype classes (low-YP, intermediate-YP, high-YP) from three environments (Kernen 2008, SC 2007, SC 2008), using PROC MIXED.

Random Effects	**KWT**			**Lutein**			**Zeaxanthin**	
	Ker 08	**SC 07**	**SC 08**	**Ker 08**	**SC 07**	**SC 08**	**SC07**	**Ker 08**
Rep	0.181	0.294	0.046	11.31	0	0	0	0
Residual	12.8***	3.77***	6.1***	862.6***	908.0***	104.1***	2.829***	1.794***
Fixed Effects								
Genotype	3.07*	4.21*	3.50*	95.1***	175.2***	164.6***	12.03***	15.51***
Sampling Date	841.7***	123.8***	845.4***	221.8***	21.55***	154.1***	79.31***	101.7***
Genotype*Sampling Date	0.76	0.37	1.29	37.34***	10.9***	23.25***	0.44	7.73***

Random Effects	**α-carotene**			**β-carotene**		
	Ker 08	**SC 07**	**SC 08**	**Ker 08**	**SC 07**	**SC 08**
Rep	0	0.012	0	0.117	0.023	0.002
Residual	11.611***	0.121***	0.24***	13.27***	0.555***	0.415***
Fixed Effects						
Genotype	11.37***	62.03***	71.53***	7.56***	1.19	37.91***
Sampling Date	23.31***	99.81***	124.9***	22.67***	16.94***	124.5***
Genotype*Sampling Date	4.06***	33.04***	60.17***	2.39*	0.80	20.97***

*p<0.05; **p<0.01; ***p<0.001

At Kernen 2008 and SC 2008, linear accumulation of lutein was observed in the high and intermediate YP groupings up to maturity with the two groups diverging statistically at 35 DAH (Figure 8). In contrast, the lutein content in the low YP accumulators peaked at 21 DAH and remained stable throughout the grain fill period (Figure 8). At SC 2007, lutein content peaked at 21 DAH in high and intermediate YP genotypes, and then slowly declined through the remainder of the grain filling period (Figure 8). This decline was greater in the high-YP group. In low pigment genotypes, lutein content was highest at 14 DAH in SC 2007 and declined significantly until 28 DAH but thereafter, the lutein content was stable (Figure 8).

In all genotypes, α-carotene and β-carotene were detected at most sampling dates except at 14 DAH, where a combination of low concentration and unresolved, co-eluting peaks hindered quantification (data not shown). The accumulation of α-carotene and β-carotene were variable among the three environments, but β-carotene accumulation was similar to α-carotene at most environments, except for Kernen 2008. At that environment, β-carotene accumulation was similar for the high and intermediate group (Figure 8). At Kernen 2008, the α-carotene content of high YP genotypes was statistically higher than the intermediate and low groups late in grain fill, but in the remaining two environments, statistical differences between the three groups was detected only at maturity (Figure 8). At Kernen 2008, a linear decease in α-carotene was observed in the low and intermediate pigment groups. At Kernen 2008 and SC 2007, the accumulation of α-carotene in the low pigment group was similar to the intermediate pigment group (Figure 8).

Zeaxanthin content (ng kernel^{-1}) was assessed in all environments, but useable data were obtained only for SC 2007 and 2008. At Kernen 2008, high concentrations of chlorophyll *a* were present in immature grain compared to the other locations. Using the initial protocol, the elution time of chlorophyll *a* was close to that of zeaxanthin (Figure 7), making it difficult to obtain precise estimates of zeaxanthin concentration. In the two remaining environments, a slight modification of the mobile phase allowed more precise estimates to be obtained. At SC 2008, zeaxanthin content increased in all genotypes 21 DAH, but in the intermediate and high groups, accumulation peaked at 28 DAH (Figure 8).

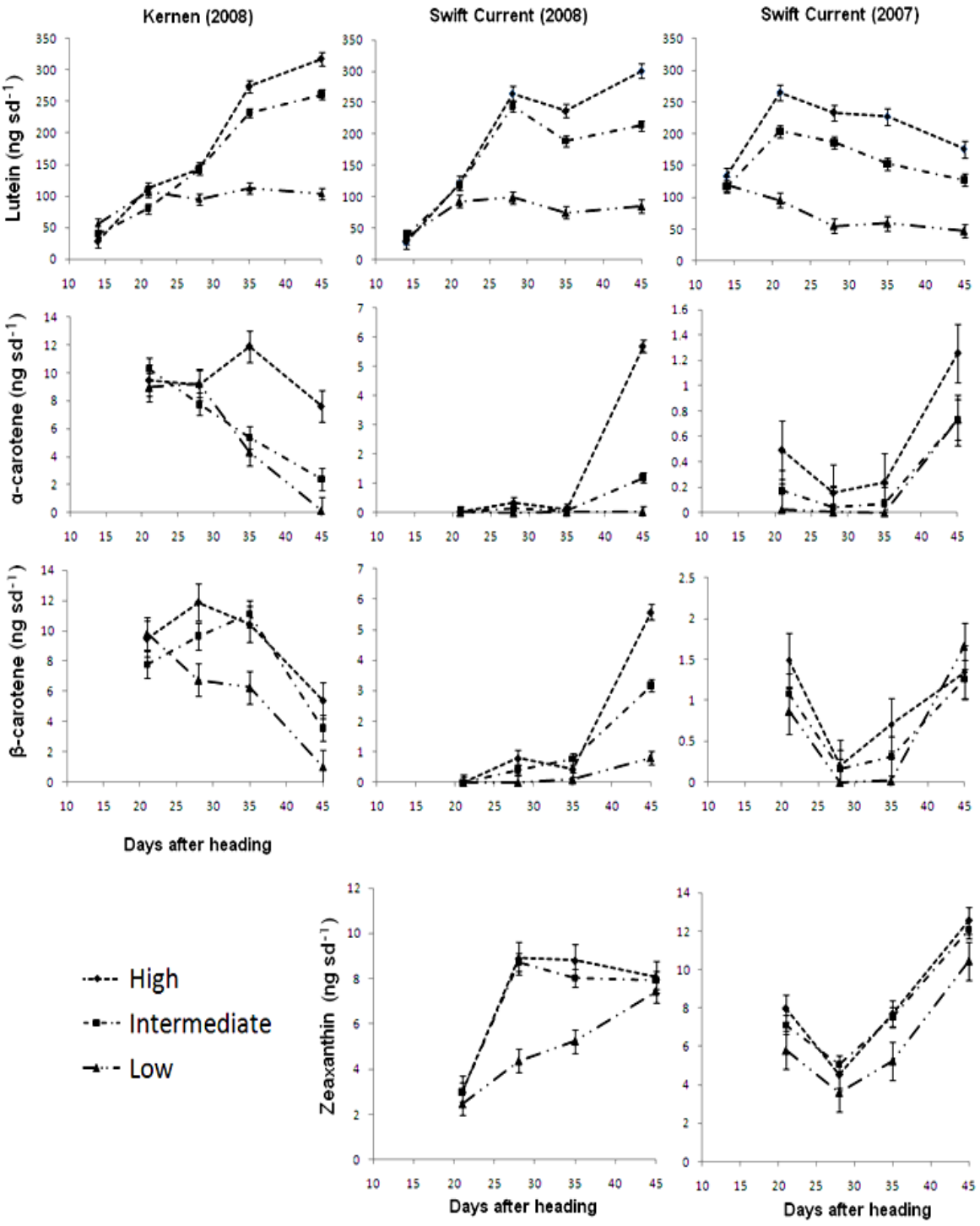

Figure 8. Accumulation of lutein, zeaxanthin and carotenes during grain fill in high, intermediate and low yellow pigment durum wheat groups in individual environments. Groups were comprised of 3 (high), 6 (intermediate) and 4 (low) genotypes. Error bars represent the standard error of the LS mean for each sampling time. Carotenoid content (dry matter basis) is expressed as ng kernel^{-1} (ng sd^{-1}).

A linear increase in zeaxanthin content was observed in the low group in that environment, but no statistical differences in zeaxanthin content were detected at maturity among the three groups (Figure 8). At SC 2007, zeaxanthin increased after 28 DAH, but no significant differences were detected among groups at any of the sampling dates.

5. DISCUSSION

5.1 Carotenoid Extraction

Because a single extraction yielded carotenoid recovery equal to the accepted AACC method for YP, this was selected along with the MeOH: DCM solvent system as the extraction method for carotenoids. Although the evaluated solvents were not significantly different at the 5% level, this solvent system was more appropriate for sample filtration and injection prior to HPLC analysis. This was because of the lower viscosity of this solvent combination and its similarity to the mobile phase. A similar protocol was also used by Abdel-Aal et al., (2007), although with water-saturated *n*-butanol as the extraction solvent. As the relative concentrations of carotenoids amongst genotypes were the primary focus of this study, a 14% loss was considered acceptable because of the good correlation between freeze-dried samples and controls. Therefore, none of the samples were freeze-dried before extraction. Immature grain samples from Kernen (2007) were not analyzed due to excessive as well as differential rates of lutein degradation confirmed with controls.

An initial saponification step was not performed, as it is time-consuming and was found by previous studies to be unnecessary for durum because of the low concentrations of lutein esters (Hentschel et al., 2002, Panfili et al., 2004). Furthermore, the procedures employed may lead to lutein degradation during extraction (Yue et al., 2005). Losses of polar xanthophylls are a potential issue during separation of carotenoids from alcohol to hydrocarbon phases as is typically performed after saponification (Craft and Soares, 1992). In retrospect, sample saponification would have removed all chlorophylls and their products (Howe and Tanumihardjo, 2006; Shi and Chen, 1999) and would have made it unnecessary to repeatedly modify the HPLC protocol.

5.2 Carotenoid Identification

In this study, lutein, zeaxanthin, α-carotene and β-carotene were conclusively identified. Unlike previous studies, we did not detect lutein esters even in subsequent HPLC runs, which contrasts with other studies that found detectable amounts of lutein

esters in durum (Atienza et al., 2007), einkorn (Abdel-Aal et al., 2007) and bread wheat (Howitt et al., 2009). However, other studies have also not detected lutein esters (Hentschel et al., 2002; Leenhardt et al., 2006). It is possible that cultivar differences may exist regarding the levels of lutein esterification, or that our method did not extract lutein esters from the sample matrix. A few samples were also analyzed using the method of Young et al., (2007) that was designed to separate lutein esters, but no lutein esters were detected (data not shown).

In mature grain, six unidentified xanthophyll-like peaks (1-4 and 7-8) were quantified with the *E*-lutein standard curve. Based on the observed hypsochromic shifts, A_b/A_{II} ratios and *Z* absorption wavelengths, peaks 3 and 4 (Figure 7) are likely *Z*-lutein isomers with single central *Z* bonds (Britton, 1995; Liaaen-Jensen and Lutnæs, 2008). This is also supported by their high correlations with lutein. On the other hand, the disparity in fine structure between the two peaks (3 and 4) suggests that the former may possess an additional *Z* bond (Britton, 1995). While the presence of 13-*Z* and 13'-*Z* isomers of lutein in durum and 15-*Z* lutein in einkorn have been reported (Abdel-Aal et al. 2007), double or poly-*Z* isomers are rarely encountered. The absorbance spectrum of peak 1 closely matches that reported for the *E*-isomers of the epoxy-carotenoids violaxanthin and antheraxanthin (Britton, 1995; Chen et al., 2004; Minguez-Mosquera et al., 1992). This is partly supported by the high fine structure observed for this peak, indicating no *Z* bonds. Its short retention time was also typical of polar epoxidated carotenoids (Chen et al., 2004). Assigning identifications to the remaining peaks (2, 7 and 8) was more ambiguous because of inconclusive *Z*-parameters. Based on the data in Table 3, peak 2 is likely to be an *E*-isomer of an unknown carotenoid (high fine structure and no *Z*-parameters).

Carotenoids with similar spectra to this peak include neoxanthin and luteoxanthin (Chen et al., 2004), although these have not been reported in durum grain. Melendez-Martinez et al., (2006) examined an isomerized mixture of lutein epoxides and reported A_b/A_{II} ratios of 46% for 13Z/13'Z lutein epoxides, and 8% for 9Z and 9'Z lutein epoxides. Given that epoxidation would not affect the relative *Z*-parameters, this supports the likelihood that peaks 3 and 4 are 13*Z*/13'*Z* lutein isomers. This also indicates that peaks 7 and 8 may be xanthophyll isomers with single non-central *Z* bonds

(e.g. 9*Z* and 9'*Z*) given the low *Z* maxima and relatively high fine structure. The dissimilar *Z*-peak shifts for peaks 7 (343 nm) and peak 8 (334 nm), combined with the higher fine structure for the former (71% compared to 66% for *E*-lutein and 100% for *E*-zeaxanthin; Table 3), suggests that peak 7 may be a zeaxanthin isomer while peak 8 may be a lutein isomer. However, correlation analysis (Table 12) suggests that both compounds are lutein isomers, given the assumption that the concentrations of *Z* and *E*-isomers are positively correlated. LC-MS analysis may be useful in future studies to examine if this assumption is true, as well as to authenticate the veracity of spectral data with regards to carotenoid identification at low concentrations.

5.3 Total Yellow Pigment

No significant genotype x environment interaction was observed for YP concentration. This permitted genotypes to be classed into three YP groups based on statistical differences. These classifications were maintained for reporting carotenoid estimates. The YP concentrations of both 'Commander' and 'Strongfield', classified into the intermediate YP group, were significantly higher in this study than those reported earlier in other environments (Clarke et al., 2005a, 2005b). Interestingly, these two genotypes were not significantly different from each other in the present study, although previous studies (Clarke et al., 2005a, 2005b) have found 'Commander' to be higher in YP. 'Kofa' and 'W9262-360*D3' also showed higher YP content than that reported earlier (Pozniak et al., 2007).

5.4 Carotenoid Expression at Grain Maturity

In this study, lutein, α-carotene, and β-carotene were significantly different among the three cultivar groupings with increasing concentrations of these as total YP increased. This confirms earlier studies where concentrations of these carotenoids in mature grain were highly correlated with YP (Abdel-Aal et al., 2007, Hidalgo and Brandolini, 2008, Hidalgo et al., 2006). The concentration of unidentified putative carotenoids was also significantly higher in lines with high yellow pigment (Table 13). This is likely the result of a greater flux of substrate through the carotenoid biosynthetic pathway in lines expressing higher total YP, as increased flux would result in higher concentrations of all carotenoids as observed in the intermediate and high YP cultivars

used in this study. Indeed we have shown that two genes coding for phytoene synthase-1 (*Psy1-A1; Psy1-B1*) the first committed enzyme to carotenoid biosynthesis (Figure 2), are associated with phenotypic differences in expression of yellow pigment (Pozniak et al. 2007; Singh et al. 2009). We have shown that an allele of *Psy1-A1* coding for a non-functional form of the enzyme is associated with low yellow pigment in 'Blackbird' (Singh et al. 2009), one of the parents of A0022&D509 used in this study. A0022&D509 expressed low YP and a corresponding reduction in lutein (Table 6) and does carry the same *Psy1-A1* allele as Blackbird. The same non-functional *Psy1-A1* allele has also been reported in many bread wheat cultivars (Zhang and Dubcovsky, 2008), which have less total yellow pigment and lutein (Adom et al., 2005; Hidalgo and Brandolini, 2008; Howitt et al., 2009) than durum wheat cultivars. The absence of both lycopene and ζ-carotene in mature grain samples indicates that enzyme activity downstream of *Psy* (i.e. *ZDS*, *LCYB*, and *LCYE;* Figure 2) is likely not rate-limiting. In contrast, the intermediate pigment cultivars Kofa, Strongfield and Commander used here have all been shown to contain functional alleles at both *Psy-A1* and *Psy1-B1* (Pozniak et al. 2007; Singh et al. 2009).

No significant differences in zeaxanthin concentration at grain maturity were detected among varieties, but zeaxanthin accounted for a larger proportion of YP in low pigment genotypes (6%) than in high pigment genotypes (2.5%). In addition, the zeaxanthin: lutein ratio was 4.6% in the high grouping, compared to 15.6% in the low pigment group. This trend of higher zeaxanthin relative to lutein contents in cultivars expressing low YP is consistent in the literature. Digesu et al., (2009) reported zeaxanthin: lutein ratios of approx. 4% in the highest-YP genotypes 'Svevo' and 'Grecale' and approximately 16% in the lowest-YP genotypes 'Messapia' and 'Timilia'. The zeaxanthin: lutein ratio was approx. 10-12% in several low-YP durum genotypes (Abdel-Aal et al., 2007, Fratianni et al., 2005; Okarter et al., 2010), as well as in bread wheat cv. 'Serio' (Hidalgo and Brandolini, 2008). Among bread wheat cultivars, ratios up to 23% have been reported in soft wheat (Roose et al., 2009) and 41% in semi-hard wheat (Okarter et al., 2010). Moore et al., (2005) reported zeaxanthin: lutein ratios ranging from 18% to 35% across eight cultivars of soft wheat. In einkorn wheats, which are usually high in total YP (Abdel-Aal et al. 2007), ratios of 4% (Hidalgo and

Brandolini, 2008) and <2% (Leenhardt et al., 2006) have been reported. The higher zeaxanthin: lutein ratio could imply that the cyclization of lycopene may be one of the major regulatory steps for xanthophyll accumulation in durum (Figure 2). This is further supported by the opposite pattern observed for lutein as a proportion of yellow pigment, i.e. 38% in low-YP genotypes and 54% in high-YP cultivars. It appears from these data that the β,ε branch of lycopene cyclization leading to the formation of α-carotene and lutein (Figure 2) is favoured over the β,β branch (β-carotene and zeaxanthin) in high-YP genotypes. As well, the zeaxanthin: lutein ratios in high and intermediate lines were not statistically different (Table 13), and this suggests that this preference is restricted to lines expressing very low levels of yellow pigment. Lycopene cyclization is controlled by two enzymes, *LCYB* (involved in both branches) and *LCYE* (involved only in the β,ε branch), so genetic variation influencing the activity or regulation of one or both of these enzymes may be responsible for the differences in ratios. Howitt et al., (2009) established an association between sequence variation in *LCYE* and lutein concentration in hexaploid wheat, so this enzyme may indeed regulate the zeaxanthin: lutein ratio. We have recently localized a gene for *LCYB* to chromosome 6B (unpublished results), the same chromosome where a major QTL for YP has been mapped (Pozniak et al. 2007). Since there are consistent differences in the zeaxanthin: lutein ratio among high and low pigment lines, understanding the genetic mechanisms behind this ratio will greatly improve our understanding of carotenoid regulation in durum.

In the carotenoid biosynthetic pathway, α-carotene is a metabolic precursor to lutein (Figure 2), and in mature grain samples, the ratio of α-carotene to lutein was highest in the high YP grouping (Table 4). This concentration of α-carotene near maturity indicates that there was still α-carotene available for conversion to lutein, and suggests that enzymes late in the pathway are not efficient in converting α-carotene to lutein. Only two enzymes, β-ring hydroxylase (*CRTR-B)* and ε-ring hydroxylase (*CRTR-E)*, are involved in converting α-carotene to lutein (Cunningham and Gantt, 1998). Perhaps alternate alleles coding for these enzymes can be found that possess higher capacity of α-carotene conversion, resulting in even greater lutein content than that observed in the high YP genotypes evaluated here. This could be of benefit because lutein and zeaxanthin are associated with the prevention of age-related macular

degeneration (Abdel-Aal et al., 2007). Xanthophylls are also more stable to heat, light and oxidants than are carotenes (Siems, 2002). This however may not be phenotypically relevant in terms of pasta colour if α-carotene and lutein equally contribute to YP. As well, a lower content of zeaxanthin relative to its metabolic precursor β-carotene was also observed in high pigment lines. Together, this suggests that in high-YP genotypes, the hydroxylation of carotenes to their respective xanthophylls may be another rate-limiting step. Because *CRTR-B* is involved in the hydroxylation of both carotenes (Figure 2), it is possible that this enzyme may be less functional in genotypes expressing higher YP. This may simultaneously explain the high carotene: xanthophyll ratios in both branches. In the case of low-YP genotypes, carotene concentrations were not significantly different from zero in most cases. This was particularly true for α-carotene.

Digesu et al. (2009) found that total unknown carotenoid components comprised about 9.5% of total yellow pigment in 52 durum accessions, which is in general agreement with our results (9-10% of total YP, after adjusting for moisture content). These unknown compounds were also suspected to be *Z* isomers of lutein/zeaxanthin or epoxy-carotenoids (Digesu et al., 2009). Given the low absorbances that are typically exhibited by *Z*-isomers of carotenoids (Britton et al., 1995), their underestimation with the *E*-lutein standard curve may partially explain the >30% discrepancy between total concentration of YP and the combined concentration of observed carotenoids (Table 2). Therefore, if these unknown compounds represent *Z*-isomers, they could constitute significantly more than the <10% of YP estimated here. Furthermore, Burkhadt and Bohm (2007) reported no discrepancy between total carotenoids and total YP when carotenoids were extracted after a semolina water treatment to enhance extraction. Those results indicate that the yellow pigment contribution from non-carotenoid components is minimal. In addition, this may show that the water used in the standard solvent for yellow pigments (water-saturated *n*-butanol; AACC 14-50) may directly aid the extraction of carotenoids from durum semolina or flour (Burkhadt and Bohm, 2007), although this solvent was not used for carotenoid extraction in the cited study. It was suggested that the association of carotenoids with more hydrophilic components (e.g. proteins) may be responsible for this phenomenon. However, this does not explain why our lutein recovery study found a greater recovery from MeOH: DCM than from water-

saturated *n*-butanol. This could suggest two possibilities: i) MeOH:DCM is also better at extracting total YP, and unknown components make up the rest of YP; or ii) a discrepancy in particle sizes of the samples submitted to total YP and to carotenoid analysis may have under-estimated carotenoid content in our study. Particle size was shown to have a large and significant effect on carotenoid recovery (Burkhadt and Bohm, 2007). Since our lutein recovery study did not include a yellow pigment assay of a similarly milled meal sample or vice versa, no direct comparison is possible. The results from this thesis, in conjunction with those of the cited study, suggest that a uniform particle size and solvent is necessary for comparing total carotenoids with total YP.

The relationship between carotenoid concentration (particularly lutein) and TKW should be considered when breeding for larger seed size, especially given the strong environmental effect on TKW in our genotypes. Abdel-Aal et al. (2007) reported a significant environmental effect on lutein concentration in einkorn wheat. It is possible that this variation was largely because of variation in seed size. When comparing Kernen 2008 to SC 2007, the larger lutein content in grain from Kernen 2008 did not sufficiently compensate for the significantly higher TKW. This resulted in lower lutein and YP concentrations at maturity. Similarly, while YP and lutein concentrations were significantly different between '2805' (high YP) and 'Kofa' (medium YP) in mature grain, the lutein content per seed was not. This is because 'Kofa' had significantly higher TKW. By convention for the methods of measuring semolina parameters, and for durum breeding purposes, a concentration-based comparison is favoured. However, we have presented both content and concentration to measure accumulation patterns. It is suggested that both TKW and concentration be taken into account when contrasting the carotenoid or YP levels of grain from different genotypes or environments. In other words, a high YP or carotenoid concentration may actually be the direct consequence of insufficient starch synthesis because of unfavourable growing conditions.

In terms of grain carotenoid physiology as reported in the literature, low-YP durum genotypes appear to be closer to hexaploid wheat than to other durum genotypes. This is reflected by the similar lutein accumulation patterns and the zeaxanthin/lutein

ratios (Hidalgo and Brandolini, 2008; Howitt et al., 2008; Roose et al., 2009). We did not examine the distribution of carotenoids in grain fractions, but other studies show similarities between hexaploid wheat and low-YP tetraploid wheat in this aspect as well (Abdel-Aal et al., 2007; Adom et al., 2005; Fratianni et al., 2005).

5.5 Carotenoid Accumulation During Grain Fill

In early grain fill, the quantification of carotenoids was difficult because chromatograms were more complex, with chlorophyll *a* and chlorophyll *b* being present in high proportions (Figure 7). As such, quantification and analysis over the grain fill period was restricted to lutein, zeaxanthin, α-carotene and β-carotene, as these could be quantified fairly reliably. Chlorophylls were not quantified, although the general trends were easily observed.

Lutein content increased significantly after mid-grain fill in medium and high-YP genotypes at Kernen and SC 2008, and this accumulation continued up to grain maturity (Figure 8). However, the rate of accumulation was less in intermediate lines after 35 DAH when compared to the high pigment group. In SC 2007, this pattern was shifted to earlier in grain fill and differences between the high and intermediate groups were observed as early as 21 DAH. These data suggest that the rate of biosynthesis of lutein in medium-YP genotypes is less compared to high-YP accumulators but the duration and timing of accumulation is consistent. This could indicate end-point regulation where after a certain amount of lutein biosynthesis is achieved, the system is down regulated. Interestingly, the period of maximum lutein accumulation in medium and high-YP groups was shifted to earlier in grain fill in environments where kernel weight at maturity was lower (data not shown). This suggests that environmental stresses that affected kernel weight had the same effect on lutein accumulation, and may explain some of the environmental variation observed in YP concentration. The reason for the significant decline in lutein content in all YP groups in SC 2007 is unknown. It is likely due to the hot and dry conditions experienced after anthesis at this environment. Compared to the 30 year average, the average monthly temperature at SC 2007 was 4.5°C warmer in July (Appendix 1). In addition, that site received only 9.8 mm of precipitation in July, compared to the 30 year average of 52 mm. Kean et al., (2007) also reported a decline in lutein content after mid-grain fill in sorghum and suggested

that a conversion of lutein to apocarotenoids or aromatic compounds may have initiated the decline. Leenhardt et al. (2006) found a significant negative correlation between carotenoid-degrading lipoxygenases and total carotenoid concentration across three wheat species. It is possible that similar degradation mechanisms may have caused the decrease in lutein content in SC 2007.

At maturity, significant positive correlations ($r>0.97$) between TKW and lutein content existed for each genotype across environments. Although YP and lutein concentrations were generally the lowest in Kernen 2008, the amount of lutein that accumulated per seed (particularly in high and medium-YP classes) was the largest in this environment. Furthermore, 'Commander' was not significantly different from the high-YP group in terms of lutein or total carotenoid content at maturity, highlighting the difference between content and concentration-based carotenoid estimates.

In contrast, the low-YP group showed differences in both rate and duration of lutein accumulation where lutein synthesis occurred early in grain fill with no appreciable increases in lutein content after 21 DAH. A similar pattern was shown in hexaploid wheat, where lutein content was constant throughout the grain fill period. At SC 2007, lutein content declined significantly in low pigment lines after our first sampling date, suggesting no biosynthesis of lutein after 14 DAH, similar to what was reported in sorghum by Kean et al. (2007). This suggests that lutein biosynthesis stops early in grain fill because of a termination in the flux of substrate through the pathway. Because no other carotenoid continued to accumulate in significant amounts, this suggests that the termination occurred early in the pathway, such as at *Psy-1*. Although zeaxanthin continued to accumulate up to the end of grain fill in all genotypes in SC 2007, these amounts were not significant in terms of total carotenoid accumulation. These results are different from those reported by Howitt et al., (2009), where zeaxanthin and its derivatives disappeared by grain maturity in bread wheat.

During grain fill, zeaxanthin accumulation patterns were quite different from those of lutein. As noted earlier, the lack of significant differences in final zeaxanthin content amongst genotypes could arise from regulation at the lycopene cyclization step or even from zeaxanthin degradation to ABA. However, the grain fill data may support the former hypothesis because no apparent decrease in total zeaxanthin content was

observed. This is different from what was observed in sorghum (*Sorghum bicolor* L.), where the accumulation of lutein and zeaxanthin occurred during the first half of grain fill and declined thereafter (Kean et al., 2007); and in hexaploid wheat, where zeaxanthin concentration decreased throughout grain fill and disappeared by grain maturity (Howitt et al., 2009).

The lower concentrations of total carotenoids and total yellow pigment in low-YP genotypes suggest that the entire pathway is also blocked upstream of lycopene synthesis, relative to high and intermediate YP genotypes. If the β,ε branch of lycopene cyclization was the only major regulatory step, more zeaxanthin (and therefore more yellow pigment) would be expected to accumulate in low-YP genotypes. This was not observed. In view of the suspected genetic variation both upstream and downstream of lycopene synthesis, no significant difference in zeaxanthin concentration or content was detected amongst genotypes from all classes (due to a compensatory effect), and consequently, no correlation with YP was evident. For the same reason, this variation would intensify differences in lutein content amongst YP classes and establish strong correlation of lutein with YP. This was indeed observed in this study.

Chlorophyll content (data not shown) declined through grain fill and disappeared by physiological maturity, in parallel with the unidentified peaks that were specific to immature grain samples. This suggests that some of these peaks may be related to chlorophyll or its byproducts such as pheophytins. There is also the possibility that they may be highly labile poly-*Z* carotenoids given their asymmetric spectra and maximal absorbance at 400-450 nm (Britton, 1995; Liaaen-Jensen and Lutnæs, 2008). The unidentified putative carotenoid peaks seen in mature grain samples co-eluted with chlorophylls in samples taken during grain fill (Figure 7), and could not be accurately quantified. Lastly, the reasons why low-YP genotypes have a greater proportion of YP unexplained by quantified carotenoids (45%) than high-YP genotypes (30%) remains unknown. No genotype x extraction interaction was seen during extraction recovery tests for carotenoids (Table 2).

6. CONCLUSIONS

The results from this thesis suggest that differences amongst YP groups involved both rate and time of carotenoid accumulation. The differences in carotenoid proportions indicate the presence of at least three rate-limiting steps in the pathway where genetic variation may exist amongst the YP classes: i) early in the pathway, possibly at the synthesis of phytoene; ii) lycopene cyclization; iii) carotene hydroxylation (Figure 9). However, the possibility that carotenoid ratios and concentrations are also regulated by downstream enzymes (such as those involved in the xanthophyll cycle and ABA synthesis) requires additional research.

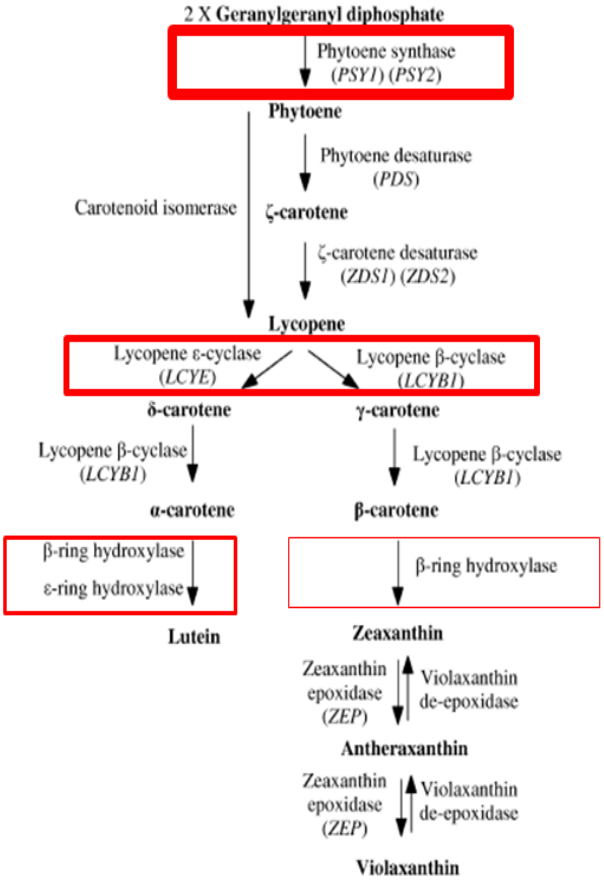

Figure 9. Carotenoid biosynthesis pathway with hypothesized rate-limiting steps (red boxes) from this study on durum wheat (adapted from Clotault et al., 2008). Line thickness approximately corresponds to the influence of that rate-limiting step on carotenoid content or composition.

Various studies have indicated the role of *Psy* and/or the *LCY* genes in influencing the concentration of YP in wheat grain. Therefore, expression profiling of these genes during grain fill may determine whether transcriptional regulation is as important in durum wheat as it is in other species. Carotenoid degradation enzymes *in planta* may also contribute to variation in carotenoid levels and these represent another class of genes to evaluate in terms of their effects on final carotenoid content in the caryopsis. Eventually, information from such studies may help in breeding for higher lutein and YP concentration while preserving the agronomic traits of registered cultivars.

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8.0 APPENDICES

Appendix 1. Average growing season temperatures (°C) and precipitation (mm) at Saskatoon (52° 10' N 106° 43' W) and Swift Current (50° 16' N 107° 44' W) in 2007 and 2008 (Environment Canada).

Month	Kernen		Swift Current	
	Temperature (°C)	Precipitation (mm)	Temperature (°C)	Precipitation (mm)
		2007		
Apr	4.8	2.0	4.5	10.5
May	11.2	46.0	11.4	37.1
Jun	15.0	131.0	15.7	56.0
Jul	21.0	22.0	22.6	9.8
Aug	15.8	17.5	17.7	19.0
Sept	10.4	24.0	11.5	23.9
		2008		
Apr	2.5	20.5	2.9	20.7
May	10.7	5.0	10.7	29.1
Jun	15.0	65.5	14.0	151.8
Jul	17.8	93.0	17.7	64.0
Aug	17.8	19.5	18.0	61.8
Sept	11.5	13.0	12.1	25.6
		30 Year Average (1971-2000)		
Apr	4.4	23.9	4.9	22.3
May	11.5	49.4	11.1	49.5
Jun	16.0	61.1	15.6	66.0
Jul	18.2	60.1	18.1	52.0
Aug	17.3	38.8	17.9	39.9
Sept	11.2	30.7	11.8	30.2